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TABLE OF CONTENTS

Chlamydospore germination, nuclear cycle, and artificial culture of Urocystis agropyri on red top.
M. J. Thirumalachar and James G. Dickson 333

The pycnidial stage of the walnut branch wilt fungus, Exosporina fawcetti ..... E. E. Wilson 340

Top necrosis, a virus disease of guar ..... W. E. Cooper 347

Cultural variation, taxonomy, and pathogenicity of Fusarium species associated with cereal root rots ..... John W. Oswald 359

Transmission of the mild streak virus of black raspberry.
N. L. Horn and M. W. Woods 377

The influence of nitrogen source on the development of stem rust of wheat ..... J. M. Daly 386

A method for the selective staining of viruses in infected plant tissues.
J. G. Bald 395

Development of the leaf spot fungus in the olive leaf ..... H. N. Miller 403

Phytopathological Notes 411

Parasitism of citrus in Florida by various species of dodder, including Cuscuta Boldinghii Urb., a species newly reported for the United States ..... L. Carl Knorr

A simple power-operated atomizer for applying small quantities of concentrated spray fluids.
A. R. Wilson and A. E. W. Boyd

A method for preserving and mounting pressed leaves.
R. S. Willison

CHLAMYDOSPORE GERMINATION, NUCLEAR CYCLE, AND ARTIFICIAL CULTURE OF *UROCYSTIS AGROPYRI* ON RED TOP

M. J. Thirumalachar and James G. Dickson

(Accepted for publication January 3, 1949)

The flag smut of grasses incited by *Urocystis agropyri* (Preuss.) Schroet. occurs on a wide range of grass hosts, several of which are of economic importance. Clinton (3) in North American Flora reported it on *Agropyron repens* (L.) Beauv., *A. spicatum* (Pursh) Scribn. and Smith, *A. smithii* Rydb., *Bromus ciliatus* L., *Calamagrostis canadensis* (Michx.) Beauv., *Elymus arenarius* L., *E. canadensis* L., *E. canadensis* var. *robustus* Mackenz. and Bush, and *E. virginicus* L. Davis (4) collected the smut near Madison, Wisconsin, on red top (*Agrostis alba* L.). Zundel and Barnhart (19) record a more extensive host range for the smut in the United States including *Poa pratensis* L., *Hordeum nodosum* L., and others. Recently more interest has been aroused in the *Urocystis* group on grasses by the findings of Fischer and Holton (7) that the flag smut of wheat (*U. tritici* Koern.) infects certain grass hosts. Since *U. tritici* and *U. agropyri* are similar and morphologically indistinct, they concluded that the two belong to the same species and so reduced *U. tritici* to a synonym of *U. agropyri*.

The life cycles of *Urocystis occulta* (Wallr.) Rab. on rye and *U. tritici* on wheat have been investigated in some detail (15, 13). In so far as the writers can determine, the chlamydospores of *U. agropyri* from grass hosts have not been observed germinating. The cross inoculations carried out by Fischer and Holton (7) were done mostly by using spores from the flag smut of wheat and, in a few cases, with spores from grass hosts. The cross inoculations were made by submerging the seed under partial vacuum in aqueous suspension of the smut spores. The appearance of symptoms on the developing plants was taken as indicating successful infections.

In the course of investigations of the stripe smuts of the grasses, the chlamydospores of *U. agropyri* from red top were germinated and the fungus was cultured on malt-extract media. The present paper is a preliminary report of the cultural types and cytology of the fungus from red top.

SYMPTOMS

The external symptoms produced by the flag smut on red top are similar to those of *Ustilago striiformis* (Westend.) Niessl var. *agrostidis* occurring on the same host. Both smuts occur on *Poa pratensis, Elymus canadensis* and *Agropyron repens* in Wisconsin, occasionally on the same plant. In the field the two can be differentiated fairly reliably by the more brownish color of the sori in *U. agropyri* and its earlier appearance in the spring.

In several bluegrass pastures near Madison, Wisconsin, the writers have noticed as high as 25 per cent flag smut infection in early spring before there was any manifestation of *Ustilago striiformis*.

Small striae, 0.5 to 1.0 mm. long, appear first, which in due course develop into elongated stripes, frequently extending the entire length of the leaves. The sori rupture the epidermis and dusty masses of spores appear on the surface of the pustules, following which the leaves become shredded longitudinally. In the newly infected plants, the leaves manifest a shredded condition and reduced culm elongation but do not show other malformations. On the other hand, the shoots regenerating from culms of plants smutted in the preceding season develop thin, narrow leaves clustered around the condensed internodes giving a witch's broom effect (Fig. 1, A). Many of the diseased plants dry out during midsummer and fail to recover following the advent of favorable conditions. The smutted plants rarely produce inflorescences.

MATERIALS AND METHODS

The fungus was studied from red top only, collected in the vicinity of Madison, Wisconsin. The smutted leaves were fixed in Karpechenko's modification of Nawaschin's fluid and formalin acetic alcohol. Sections 8–10 μ thick were cut and stained with Heidenhain's iron-alum haematoxylin with orange G as counterstain or with Newton's iodine, gentian violet. The chlamydospores were germinated and stained by the method described for rust teliospores by Thirumalachar (16). The germinating spores were transferred aseptically onto potato-dextrose agar or malt agar to secure the cultures of the fungus.

CHLAMYDOSPORE GERMINATION

As stated earlier the chlamydospores of *Urocystis agropyri* from the grasses have not been germinated previously. Schellenberg (14), Ciferri (2), Liro (11), Fischer and Hirschhorn (6), and others have reported failure. Recently Kreitlow (10), studying the smut on timothy, failed to secure germination in spite of using treatments to break the dormancy. Thirumalachar and Dickson (18) reported the first successful germination of the chlamydospores from red top and noted some of the cultural characters in vitro. The chlamydospores were fixed to the slides by wetting and drying and the slides were inverted over a water surface as described earlier. In the early experiments several of the spores developed promycelia with a whorl of sporidia at the apex after 45 days incubation at about 24° C. Pretreatment with benzaldehyde (3:2,000,000) as used by Stakman *et al.* (15) for breaking the dormancy of chlamydospores of *U. occulta* and the addition of fragments of green host tissue found useful by Noble (12) to stimulate the germination of *U. tritici* were not effective in increasing germination percentages. Satisfactory germination was obtained when the spores were fixed to the slides by repeated wetting and drying, then placed

in Coplin jars containing distilled water and incubated at 40° C. for eight hours. After this treatment the slides were removed and inverted over the water surface in the usual manner; several of the spores had germinated after 5 days. Long promycelia with a whorl of three to four sporidia characteristic of the Tilletiaceae developed from the germinating spores (Fig. 2, b to d).

CYTOLOGY OF THE FUNGUS

Stained material of various stages of spore germination showed the nuclear behavior typical of the smut fungi. The fusion nucleus migrated into the promycelium (Fig. 2, b) and there divided twice to form four haploid nuclei. After the nuclear divisions, three to four sporidia developed at the apex of the promycelium and a single haploid nucleus migrated into each sporidium (Fig. 2, c to e). In instances where three sporidia were formed, the supernumerary nucleus either remained within the promycelium or infrequently migrated into one sporidium (Fig. 2, f) to initiate the dicaryophase. After sporidial formation, conjugation tubes were formed between the compatible pairs of sporidia while still attached (Fig. 2, d to f). After sporidial conjugation the dicaryotic nuclei migrated into the infection hypha produced, usually from the conjugation tube. The infection hyphae elongated rapidly with a tendency to become aerial (Fig. 1, B). In many instances the promycelia developed two sporidium-like structures which elongated rapidly into slender hyphae (Fig. 2, g). These were usually binucleate which indicated direct migration of paired nuclei or the initiation of the dicaryophase directly without sporidial conjugation. This type of dicaryophase initiation has been described for *Urocystis occulta* by Stakman *et al.* (15) and for *Cintractia montagnei* by Rawitscher (13). When the germinating spores were submerged in water, the promycelium elongated indefinitely, septa developed at the base, and the protoplasmic contents migrated towards the apex.

Chlamydospore development within the host was studied in microtome sections of the smutted leaves. The mycelium and spore formation were similar to those described by Stakman *et al.* in *Urocystis occulta*. The spores formed from the dicaryotic mycelium which concentrated in a closely packed mass replacing the mesophyll cells between the vascular bundles in the leaf. The fertile cells with dense cytoplasm were surrounded by vacuolate hyphal cells, with nuclei in various stages of degeneration, that later constituted the sterile cells of the spore ball (Fig. 2, a). The mature spores were composed of one to three fertile cells surrounded completely by hyaline to pale cinnamon yellow sterile cells. The fertile spores were thick-walled and reddish-brown. Nuclear fusion occurred in the fertile cells in the early stages of spore formation.

ARTIFICIAL CULTURE OF THE SMUT

Cultures of *Urocystis agropyri* were obtained by transferring germinating chlamydospores aseptically onto potato-dextrose agar or malt agar.

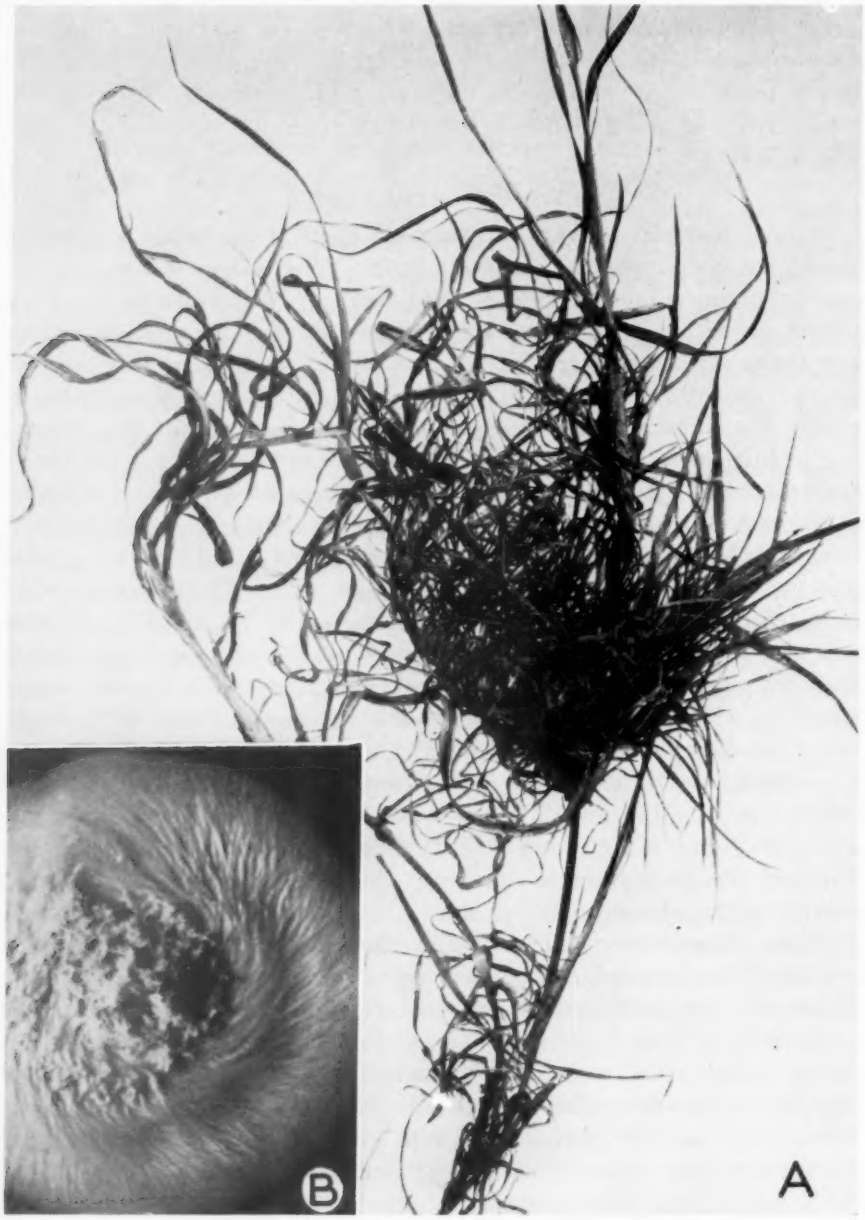

FIG. 1. *Urocystis agropyri* on red top.
A. Witch's-broom type of growth from culms of smutted older plants. Natural size.
B. Mycelial culture on malt agar. Natural size.

In other instances, the germinating spores were distributed on water agar and the single spores were picked out by Keitt's cutter and transferred to nutrient media. Lacking well developed aerial mycelium, the developing colonies were smooth, glistening, and flat. At temperatures between 20°

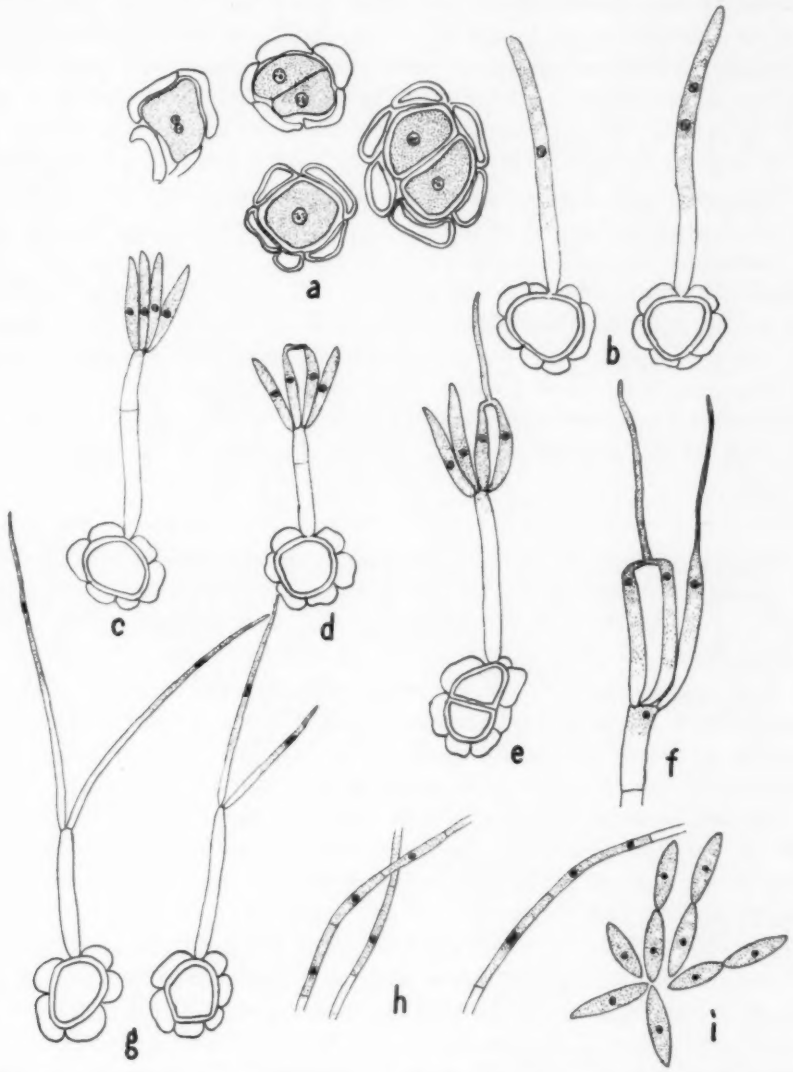

FIG. 2. Chlamydospore development, spore germination, and mycelial variants in *Urocystis agropyri*. (Camera-lucida drawings ×600 except f ×1200.) a. Young and mature sporeballs. b. Early stages of spore germination. c. Promycelium with four sporidia. d, e, and f. Conjugation of sporidia. g. Direct migration of the paired nuclei into branches. h. Dicaryotic and haploid hyphal cells. i. Sporidia from sectors in mycelial cultures.

and 24° C. a counter-clockwise spiral contour was pronounced on the colony surface (Fig. 1, B).

In the mycelium developed in culture, the young hyphae were multinucleate but showed the pairing of the dicaryotic nuclei. Septa were formed soon resulting in the characteristic dicaryotic mycelium. In several of the sectors evident in culture, a large number of uninucleate hyphae were found associated with the dicaryotic mycelium (Fig. 2, h). In some of these subcultures the stages in the separation of the paired nuclei and subsequent formation of uninucleate cells were observed. Such dediploidization of the dicaryotic hyphae has been described in several other smuts such as *Ustilago longissima* (1), *U. hordei* and *U. kolleri* (5), *U. zeae* (8), and *U. striiformis* var. *agrostidis* (Thirumalachar and Dickson, in press).

In some instances small glistening white colonies with convoluted surface were produced as sectors in the cultures of the dicaryotic hyphae which on examination proved to be sporidial in nature. The sporidia were ovate-ellipsoid, hyaline, and thin-walled (Fig. 2, i). These colonies developed rapidly by yeast-like budding characteristic of smut sporidia. They resembled very closely the sporidial colonies obtained in the subcultures of the dicaryotic mycelia of *Ustilago striiformis* var. *agrostidis*. No conjugations between sporidia were obtained. However, at temperatures between 20° and 24° C. several mycelial sectors reappeared in these sporidial colonies.

DISCUSSION

The morphology of spore germination and the nuclear cycle in *Urocystis agropyri* on red top was similar to the general type recorded for other species of *Urocystis*. The mycelium in the host was dicaryotic and the nuclear fusion occurred within the young chlamydospore. Assuming from the cytological evidence that the reduction division occurred within the promycelium, the dicaryophase was initiated again by the conjugation of sporidia or by the direct migration of two haploid nuclei into the slender branches developed under some types of germination.

The development of the mycelium and sporidia on media was similar to that of *Ustilago striiformis* var. *agrostidis* occurring on the same host. There was a marked parallelism between the two species in mycelial development and in the production of sporidial sectors by dediploidization. Typical chlamydospores of *U. agropyri* have not been observed in culture although spore-ball-like structures without differentiated fertile and sterile cells were present in old cultures of the dicaryotic mycelium. Thick-walled, resting cells occurred frequently in old cultures. Kniep (9) reported the formation of true chlamydospores in *U. anemones* in culture or the development of the fungus from chlamydospore to chlamydospore in vitro.

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THE PYCNIDIAL STAGE OF THE WALNUT BRANCH WILT FUNGUS, EXOSPORINA FAWCETTI

E. E. Wilson

(Accepted for publication January 3, 1949)

In 1947 the writer (7) proposed the name *Exosporina fawcetti* sp. nov. for a fungus causing branch wilt in Persian walnut trees (*Juglans regia* L.). This fungus, also found by the writer on branches of lemon (*Citrus limonia* Osb.), California black walnut (*Juglans hindsii* Jep.), and European chestnut (*Castanea sativa* Mill.), proved to be identical with one reported by Fawcett (3) from California on frost-injured branches of orange (*Citrus sinensis* Osb.) and grapefruit (*C. grandis* Osb.). Fawcett (3) noted the similarity between his fungus and a fungus described as *Torula dimidiata* by Penzig (6) in 1887. Recently Calavan and Wallace (2) proved isolates of *E. fawcetti* from citrus to be pathogenic to grapefruit trees.

The generic classification of *Exosporina fawcetti* was based on its production of brown, single-celled, irregularly shaped conidia (arthrospores) by segmentation of numerous parallel, closely packed hyphae, which arose from a slight hypostroma beneath the bark periderm. Such a fruiting structure was regarded as a sporodochium. On cornmeal agar the fungus produced an elaborate multicellular hypostroma which bore the arthrospores on its surface. In one series of cultures, one to two locules developed in this stroma-like body (Fig. 1, A). The locules were filled with structures resembling pycnospores, but as these never matured they were not identified with certainty. Subsequent attempts to produce a pycnidial stage in artificial media and on steam-sterilized walnut twigs failed.

On another occasion, stromata with pycnidial locules were found scattered among the arthrospores of *Exosporina fawcetti* on one small walnut twig. Since all but a few of the pycnospores had been discharged none was obtained for culturing. Extensive search for two years failed to reveal another case of a similar nature.

The writer recognized the similarities between his fungus and the toruloid stage of *Hendersonula toruloidea*, which was described by Nattrass (4) in 1933, but he did not feel justified in reporting it under that name.

In December, 1947, at Davis, California, numerous black carbonaceous pycnidial stromata were found on cankers developed by inoculating the trunks of young Persian walnut trees with *Exosporina fawcetti* (Los Molinos isolate) in May, 1947 (Fig. 1, C). A few arthrospores typical of those of *E. fawcetti* were clustered over and around the stromata.

In September, 1948, pycnidia and pycnospores identical with those mentioned above were found on pieces of walnut twigs that had been surface-sterilized with mercuric bichloride, placed in culture tubes containing a small amount of water, and inoculated with *Exosporina fawcetti* on Novem-

ber 19, 1947. The tubes were kept in the laboratory near a north window and sterile water was added occasionally.

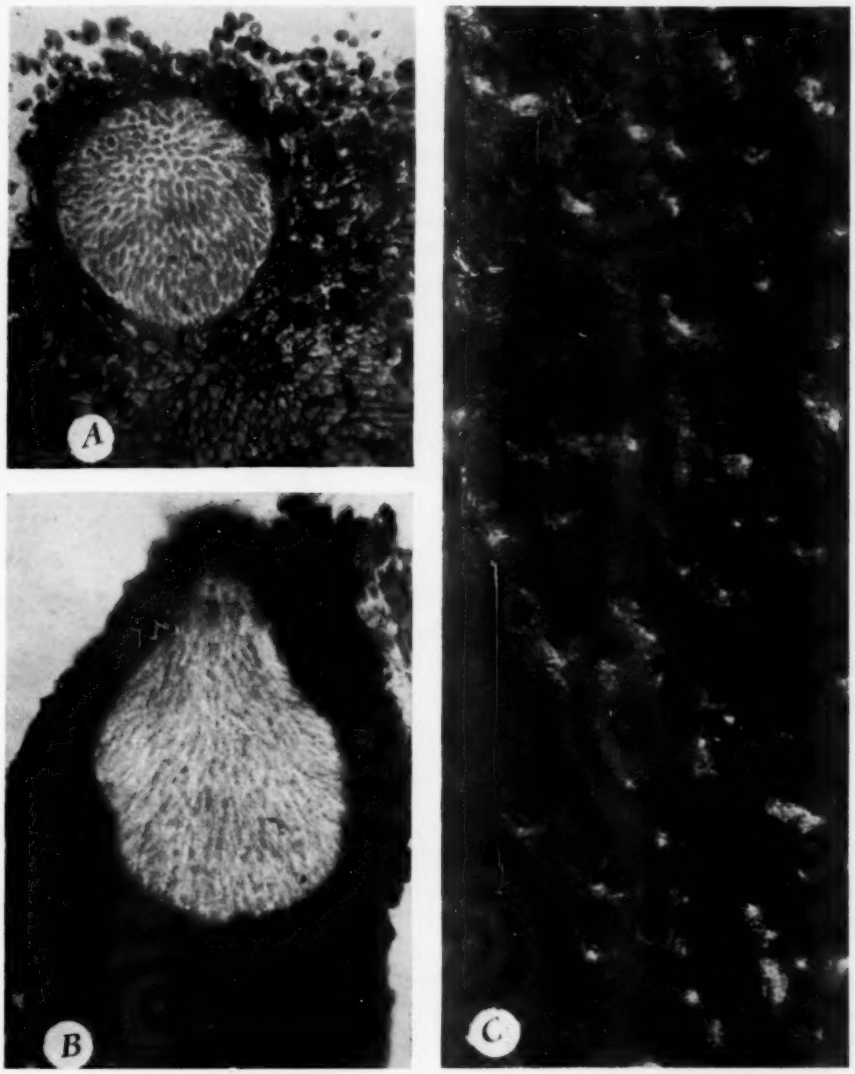

FIG. 1. A. Locule in a stroma-like structure produced by *Exosporina fawcetti* on cornmeal agar and filled with what appear to be pycnospores. The free arthrospores at top of photograph were produced on the surface of the stroma-like structure. B. Single pycnidium on a basal stroma which developed beneath the periderm of a walnut twig inoculated with *E. fawcetti*. C. Stromata breaking through the periderm of a walnut twig inoculated with *E. fawcetti*.

DESCRIPTION OF THE PYCNIDIAL STAGE

One to seven black pycnidia were partially embedded in each stroma. The necks of the pycnidia were separate and arose to a height of 50 to 70μ

(Fig. 1, B, Fig. 2, D). Stromata varied between about 125 to 500μ in diameter and 150 to 225μ high. The flask-shaped pycnidial locules (Fig. 1, B), measuring 75 to 100μ in diameter and 100 to 150μ long and occurring at different levels in the stroma, were without walls differentiated from the rest of the tissue.

The non-septate, hyaline to slightly yellow, elongate, guttulate pycnospores (Fig. 2, A) were borne on slender, flexuous sporophores measuring 3 to 4μ broad and 10 to 15μ long. The spores measured 3.2 to 6.5μ wide (average, 5.3μ) and 11.9 to 16.0μ long (average, 14.6μ).

When the bark was wetted, pycnospores were extruded from the pycnidia in white cirri (Fig. 2, D). Within 24 hours at room temperature (23°–25° C.) the cirri became somewhat darkened and after 48 to 72 hours, jet black. The content of many of the spores from partially discolored cirri

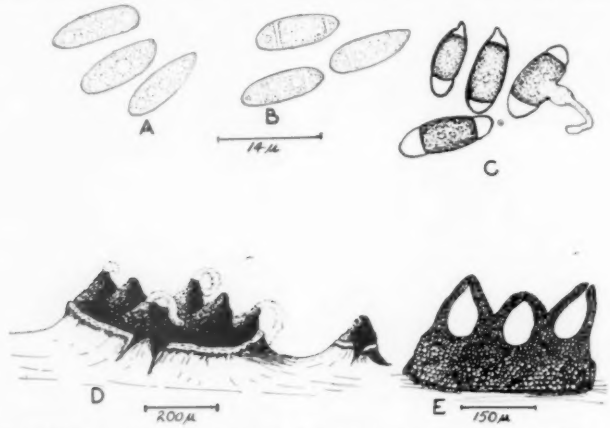

FIG. 2. Pycnidia and pycnospores of *Exosporina fawcetti*. A. Non-septate hyaline pycnospores from pycnidium. B. Septate pycnospores about 48 hours after extrusions from the pycnidium. The spores at this time are sub-hyaline. C. Pycnospores about 72 hours after extrusion from pycnidium. The center cell is darker than the terminal cells and its content is rounded at the ends, causing the cross-walls to bulge into the end-cells. D. Habit sketch of two stromata, one with six pycnidia, the other with one. Pycnospores are extruded in white cirri which soon turn black. E. Vertical section through a stroma with three pycnidial locules.

were separated into three parts (Fig. 2, B), but under the ordinary microscope no walls could be distinguished at these separations. Under cross Nicol prisms of the polarizing microscope, however, these areas of separation exhibited the anisotropic or double refraction phenomenon, indicating that cellulose was present.

Most pycnospores from jet black cirri were two-septate, with thicker walls than non-septate spores (Fig. 2, C). The contents of the center cells, which were darker in color than those of the terminal cells, were rounded at the ends. Consequently, the septa, which could be detected under crossed Nicol prisms, bulged into the end-cells. In many cases the spore wall around the terminal cells was noticeably wrinkled and sometimes collapsed inward.

Although the hyaline, non-septate pycnospores germinated readily in

double-distilled water at 25° C., only a very few of the three-celled colored spores germinated under identical conditions. Later it was found that from 60 to 70 per cent of the colored spores germinated when placed in water drops on the surface of potato-dextrose agar.

CULTURAL FEATURES

Single hyaline, non-septate pycnospores were isolated and placed on potato-dextrose agar. After incubating about a week at 25° C., mycelium extended across the plate, and a pulverulent surface growth, which was first white then greenish-black to black, developed. This surface growth was composed of numerous branched, aerial hyphae which become septate throughout their length, producing dark brown, irregularly spherical, ovoid, or wedge-shaped segments. The entire structure broke apart into single-celled segments (arthrospores) when placed in water. These germinated readily on water agar at 25° C.

On potato-dextrose agar the fungus was characteristic for *Exosporina fawcetti*. The arthrospores which developed from single pycnospores averaged 4.6 μ and 7.3 μ long, the mean dimension being 5.9 μ. This is slightly, but probably not significantly, larger than the mean dimensions of 5.1 to 5.5 μ obtained in measuring the arthrospores of various isolates of *E. fawcetti*.

The same fungus grew out of chips of wood removed aseptically from beneath the cankers on which the pycnospores were produced. Within two or three weeks at room temperature, sporodochia ½ to 1 mm. in diameter and bearing numerous arthrospores on the surface developed on chips of wood planted on potato-dextrose agar. No evidence of a pycnidial stage, however, was found in such cultures grown for several months.

EFFECT OF TEMPERATURE ON GERMINATION OF SPORES

According to earlier tests (7), optimum germination of the arthrospores and growth of the mycelium of *Exosporina fawcetti* occurs between about 30° and 33° C., whereas these processes are much reduced at 16° C. or below. Judging from the data in table 1, the optimum germination of non-septate pycnospores from the host and arthrospores from artificial media also occurs at 30° C. or more.

TABLE 1.—*The germination of pycnospores and arthrospores of Exosporina fawcetti at various temperatures*

Temperature in degrees centigrade	Percentage of spores germinating after 23 hours in water drops	
	Pycnospores	Arthrospores
6	0	0
9	14	0
15	76	44
21	80	46
25	87	60
30	98	73

PATHOGENICITY TO PERSIAN WALNUT, PEACH, AND APRICOT TREES

In June, 1948, the Los Molinos isolate of *Exosporina fawcetti* and an isolate obtained from a pycnospore were inoculated in branches of the Franquette variety of walnut, the Tilton variety of apricot, and the Phillips Cling variety of peach. Pieces of agar bearing mycelium and arthrospores of the respective isolates were inserted into cuts made in the sapwood. Waxed paper was placed over the cuts and bound with adhesive tape.

Within three weeks the two isolates produced similar dark brown to black necrotic areas 2 to 3 inches long in the bark and sapwood of the three hosts. Gum exuded profusely from inoculated cuts in apricot and peach branches. The toruloid conidial growth, which was recovered in isolations from cankers produced by each isolate, proved identical in all respects.

COMPARISON OF THE FUNGUS WITH HENDERSONULA TORULOIDEA NATTRASS

In 1933 Nattrass (4) reported isolating *Hendersonula toruloidea* from apples, peaches, and apricots suffering from a die-back in Egypt. This fungus was not considered the primary cause of the die-back and no inoculations were made to test its pathogenicity. It will be seen from the following review of Natrass' description that the California fungus closely resembles *H. toruloidea* in morphological and cultural features.

On artificial media, for example, *Hendersonula toruloidea* produced at first a white pulverulent aerial growth which eventually became olivaceous black. Short, branched, convolute hyphae rising from the agar segmented into chains of "thallospores" (arthrospores). When mature, the thallospore segments were citrine drab to bone brown in color, globose, ovoid, or subcuneiform in shape, sometimes 1-septate, 4 μ broad and 4 to 10 μ long. Optimum temperature for growth of the fungus was 30° to 35° C.

This, the "Torula" stage, was the only one Nattrass (4) found on artificial media, but on inoculated cotton and willow twigs the fungus produced black carbonaceous pycnidia, single or in small aggregates, on a subicle or rudimentary stroma.

Two years after first isolating the fungus Nattrass (4) found its pycnidial stage in nature. One or more pycnidia immersed in a stroma became erumpent through the bark periderm. Pycnidial locules, which occurred at different levels in the stroma, were not surrounded by a wall differing from the stromatic tissue.

When wetted the pycnidia extruded white cirri which contained oblong, single-celled, hyaline to slightly greenish pycnospores, 4–5 μ wide and 12-13.5 μ long. After a time the cirri turned jet black and the pycnospores became two-septate. The center cells of such spores were dark brown, the terminal cells subhyaline. These spores were said to germinate less readily than hyaline spores.

In nature the arthrospores were borne in chains on a basal plate of fungus tissue. Though Nattrass (4) did not follow the development of

these plates, he believed they later enlarged into stromata which produced pycnidia.

Dr. E. W. Mason, Mycologist of the Commonwealth Mycological Institute, Kew, England, kindly sent the writer a specimen of *Hendersonula toruloidea* which Natrass deposited at that institution in 1930. The material was said by Dr. Mason to be a culture of the fungus on twigs.

Both arthrospores and pycnidia were present on this material. The former, though somewhat more regularly sphaeroid or ovoid than those of the California fungus, were about the same size (4.9 to 6.9μ), average mean diameter 5.6μ. Pycnospores from the pycnidia were hyaline, guttulate, elongate, 3.3 to 4.3μ broad (average 3.9μ) and 9.9 to 13.6μ long (average 11.6). No dark three-celled spores were found. The one- or two-loculate stromata were very similar in shape to those produced by the California fungus (Fig. 1, B; Fig. 2, D and E).

Other reports of Hendersonula toruloidea. Nattrass (5) later found *Hendersonula toruloidea* on citrus trees in Cyprus. Fawcett (3, p. 218 and 222) reports that in California A. J. Olson isolated a fungus resembling *H. toruloides* from frost-injured citrus branches following a freeze in 1931. Upon inoculation, the fungus produced lesions in citrus branches and black masses of hyphae and spores beneath the periderm.

In 1937 Bates (1) reported isolations of *Hendersonula toruloidea* from orange fruit in southern Rhodesia. On such fruit it produced a dark brown pliable decay. Fawcett (3, p. 464) also obtained a decay of oranges and lemons inoculated with the fungus.

CONCLUSIONS AND SUMMARY

In December, 1947, at Davis, California, pycnidial stromata developed beneath the bark of young Persian walnut trees inoculated some months earlier with *Exosporina fawcetti*, the pathogen of walnut branch wilt. Isolates from pycnospores produced a toruloid conidial (arthrospore) stage which proved similar to *E. fawcetti* in morphology, temperature requirements, and pathogenicity to walnut, peach, and apricot trees.

Nattrass, in 1933, described and named a fungus, *Hendersonula toruloidea*, which produced both a toruloid and a pycnidial stage on apples, peaches and apricots in Egypt. Judging from Nattrass' descriptions and illustrations, the California fungus resembles this organism closely. Studies of type specimens of *H. toruloidea* confirmed this view. It is concluded, therefore, that *Exosporina fawcetti* is the conidial stage of *H. toruloidea*.

Under the rules of nomenclature the fungus should be designated *Hendersonula toruloidea* providing Nattrass was first to describe it. Both Fawcett and the writer, however, have mentioned the similarity between the toruloid stage of the California fungus and that of *Torula dimidiata* Penz. described from Italy in 1887. Since Penzig's description is meager, sub-

stitution of the species *dimidiata* for *toruloidea* does not seem justified at this time.

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TOP NECROSIS, A VIRUS DISEASE OF GUAR[1]

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INTRODUCTION

Guar, *Cyamopsis psoraloides* DC., is a native of India, where it is extensively grown as a food and forage crop. Although grown experimentally in the United States since 1903 (8), it recently became of importance as a source of a mucilaginous material found in the seed. Guar normally yields between 500 and 1000 pounds of seed per acre in California and Arizona where most of the commercial crop is grown (7).

In July, 1944, K. Starr Chester observed a stipple necrosis of the young leaves of guar in an experimental planting at Stillwater, Oklahoma. Preliminary infection studies indicated that the disease was caused by a virus. A search of the literature failed to disclose a disease of this nature affecting guar. Following preliminary infection studies, the disease was reported as a new virus disease of guar (2), and was later given the name "top-necrosis" (16).

When first observed on July 7, 1944, less than 0.1 per cent of the plants had definite symptoms. The leaf symptoms were yellowing, stunting, occasionally vein-clearing or a faint oak-leaf pattern, and still more rarely a light chlorotic mottling. By careful observation one could discern a stipple of very small chlorotic depressions on the leaflets. As the leaves became older these chlorotic areas became necrotic. Unequal interference with growth throughout the leaflet often resulted in skewness or rugosity. Many of the more mature leaves became bronzed, with marked intercostal chlorosis.

A count of plants with definite symptoms showed that 2.2 per cent were infected on August 18. Some of the more severely affected plants now had dark brown to black lesions on the upper portion of their stems and on the petioles. These lesions became more pronounced as the season advanced, causing a general necrosis of the stem and growing point of the plant, severe abscission of the younger leaves, and finally, bare stems with only a few chlorotic lower leaves (Fig. 1). By September 26, 40 per cent of the plants were thus affected, and a total of 90 per cent were showing definite symptoms. At this time the disease was causing an estimated loss of at least 75 per cent (2). The rapid increase in the number of af-

[1] Based on a thesis presented to the Graduate Faculty of Oklahoma Agricultural and Mechanical College in partial fulfillment of the requirements for the Degree of Master of Science.

[2] The author wishes to extend his appreciation to Dr. K. Starr Chester for his direction and valuable criticism during the course of the investigation and in the final preparation of the manuscript.

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fected plants may have been due to development of symptoms in plants previously carrying the virus in a masked condition, or to an increase in transmission by some insect vector.

The object of this study was to identify the virus causing the disease by determining its host range and the symptoms produced on the susceptes, and to investigate its infectivity, transmissibility, thermal inactivation point, dilution end point, and other biological characteristics.

FIG. 1. Four guar plants with various expressions of top-necrosis. Note the blackened necrotic stem of the plant on the left, the defoliation and the curled young leaves of plants in center, and acutely bent stem tip of the plant on the right.

SYMPTOMS OF THE DISEASE ON VARIOUS SUSCEPTS

The following descriptions apply to the disease on several species of plants that were artificially inoculated. Particular attention has been given to those plants commonly used in the diagnosis of virus diseases of legumes. The rubbing method was used almost exclusively in making the inoculations, *i.e.*, the infected plant sap was applied to the upper surface of the leaves with swabs of soft cotton cloth.

On July 12, 1944, 100 plants, 10 each in 10 randomized locations, in the field planting where traces of the disease were first observed, were inoculated with expressed juice from plants in the same field. The first symptoms were observed 9 days later and consisted of a fine chlorotic stipple of the young developing leaflets. By the 12th day a necrotic stipple was the common symptom, with 75 per cent of the plants showing infection. During this time the mean temperature was 80° F. as recorded by a weather station ¾ mile away (6); however, occasional readings showed the actual field temperatures to be much higher. The inoculated plants had 100 per cent infection after 15 days, with most of the plants showing both local and systemic

symptoms, while infection of the noninoculated plants in the field did not exceed 1 per cent. Two months later, 31 per cent of the stem tips were dead.

Guar plants growing out-of-doors in shaded flats were inoculated on August 16. Fifteen per cent of the plants showed well-developed local lesions within 4 days, and systemic symptoms similar to those that developed in the field-grown plants appeared within 6 days. Several different inoculation tests in the greenhouse during the fall and winter gave similar results.

Eight young healthy tobacco plants, *Nicotiana tabacum* L. var. Turkish, growing out-of-doors in 5-in. pots, were inoculated on September 21. Four of these plants developed from 1 to 4 local necrotic lesions each. The initial diameter of the lesions ranged from 3 to 5 mm. The lesions consisted of a thin necrotic ring enclosing slightly chlorotic tissue, which after a time became necrotic throughout.

Under normal greenhouse conditions a total of 25 tobacco plants, which were inoculated at different times, failed to produce symptoms, and subinoculations failed to indicate that the virus was present in masked condition. However, freshly inoculated plants placed under a moistened sheet, where the temperature averaged approximately 68° F., developed watersoaked rings in 8 days. These rings soon became necrotic, surrounding green centers, which slowly became necrotic throughout. No systemic symptoms developed, nor were the young leaves carrying the virus, so far as could be determined by subinoculations. It should be pointed out that several environmental factors were changed when the plants were placed under the moist sheet.

Twelve succulent tobacco plants growing in a floor bed of the greenhouse were topped to a height of 18 in. and inoculated on March 10. Local lesions were observed on 5 of the plants 10 days later, and by the 17th day 11 plants had developed systemic as well as local symptoms. A comparable inoculation made March 11 with tobacco ringspot virus also resulted in severe infections. During this period the greenhouse temperatures ranged between an average daily maximum of 93.5° F. and an average daily minimum of 71.8° F., with a mean of 80.6° F. Probably the vigorous growth of the plants, or the effect of topping them, overcame the inhibitory effect of the high temperatures (14). Later 25 other tobacco plants were inoculated with the guar virus. Local symptoms appeared on 56 per cent, and systemic symptoms on 16 per cent of these plants.

Inoculated Jimson weed, *Datura stramonium* L., developed very small, indefinitely outlined chlorotic areas on the inoculated leaves within 6 days; two days later these had changed to necrotic rings or continuous lesions from 0.5 to 1.0 cm. in diameter. The systemic infection consisted of chlorotic or occasionally necrotic rings or lesions, which by enlargement developed into a chlorotic mottle. The virus could be recovered by sub-inoculations.

The first inoculations of cowpea, *Vigna sinensis* Endl. var. Clay, gave a very small percentage of locally infected plants. In an attempt to find a local lesion host suitable for quantitative studies of the virus, 5 varieties of *V. sinensis* Endl., namely, Clay, Black Eye, New Era, Brown Sugar Crowder, and Chinese Red, and *V. catjang* (Brum.) Walp. var. Catjang were inoculated. A very fine washed sand was used as an abrasive in this test. The varieties Clay, Black Eye, and Catjang developed numerous continuous red lesions 0.5–4.0 mm. in diameter within 5 days after inoculation. The varieties New Era, Brown Sugar Crowder, and Chinese Red formed reddish necrotic rings with more or less normal centers. The diameters of these rings varied with the different varieties, from 0.5–3.0 mm. for Brown Sugar Crowder to 2.0–5.0 mm. for New Era. In all varieties except Clay, necrosis spread along the leaf veins from the point of initial infection. The intercostal tissue of the leaf soon became chlorotic. The leaves had drooped at the pulvinus by the 6th day. Then necrosis, which had spread to the stem by way of the petiole, resulted in death of the plant within 9 to 12 days after inoculation.

Several varieties of bean, *Phaseolus vulgaris* L., were tested and all proved to be only systemically susceptible. In order to make further comparisons, more varieties were tested. The seeds were furnished by the Corneli Seed Company, St. Louis, Missouri, and Asgrow Seed Company, Memphis, Tennessee. The relative number of plants infected and the severity of the disease are shown in table 1. Severity of the systemic symptoms is rated in one of three classes. Class 1 indicated a chlorotic stipple or a very mild necrotic stipple, usually occurring along the margins of the leaflets, sometimes resulting in rugosity. Typical of this group were the Kentucky Wonder selections. Class 2 was characterized by a marked necrotic stippling of the young leaflets which occasionally abscised. An occasional stem tip was killed, especially if the plants were inoculated before the first trifoliate leaves developed. The variety Full Measure typifies this group. Class 3 was similar to the preceding, but with more leaf necrosis and abscission, as in the variety Plentiful. Death of the growing point was common.

The incubation period for systemic infection varied from 6 to 10 days. The local symptoms consisted of necrotic lesions occurring within 4 to 6 days. Necrosis rapidly spread to the vascular system of the leaf and petiole and resulted in a drooping of the leaf at the pulvinus or in a downward curvature of the entire petiole. Even though the percentage of plants developing local symptoms was usually low, the virus frequently killed the plant.

Five varieties developed both local and systemic symptoms. Two varieties, Ideal Market and Genuine Cornfield, developed reddish-brown rings at the points of infection; veinal necrosis spread from this. Similar, although more marked, local lesions appeared on the variety Yard Long, *Vigna sesquipedalis* (L.) W. F. Wright. The symptoms on this variety are characteristic for this genus as described above.

TABLE 1.—*Reaction of bean varieties to guar virus*

Variety and source[a]	Plants with systemic infection		Plants with local infection
	Number infected	Disease rating[b]	Number infected
Tennessee Green Pod (A)	7 of 20	3	
Burpee's Stringless Green Pod (A)	7 of 8	2	
Asgrow's Stringless Green Pod (A)	9 of 10	2	
Landreth Stringless Green Pod (C)	9 of 10	2	
Giant Stringless Green Pod (A)	9 of 10	2	
Giant Stringless (C)	5 of 9	2	
Tendergreen (C)	4 of 10	2	
Tendergreen, New Style (C)	7 of 10	2	
Florida Belle (A)	7 of 21	2	
do (C)	10 of 10	2	
Keystonian (C)	7 of 9	3	
Full Measure (A)	9 of 10	2	
do (C)	8 of 10	2	
Bountiful (A)	5 of 10	2	
do (C)	2 of 10	3	
Plentiful (A)	10 of 10	3	
do (C)	8 of 8	3	
Commodore (C)	2 of 10	2	
Fordhook Favorite (C)	5 of 7	2	
Dwarf Horticultural (C)	4 of 10	3	
Streamliner (A)	5 of 9	2	
do (C)	7 of 9	1	
Asgrow Stringless Black Valentine (A)	8 of 10	2	
Stringless Black Valentine (C)	10 of 10	3	
Black Valentine Round Pod (C)	3 of 10	2	
Red Valentine (A)	3 of 10	3	
do (C)	4 of 10	2	
Red Valentine Stringless (C)	8 of 10	2	
Sure Crop Black Wax (C)	20 of 30	3	
Pencil Pod Black Wax (C)	22 of 22	2	
Davis Stringless Wax (C)	14 of 28	2	
Brittle Wax (C)	17 of 24	3	
Golden Wax Improved (C)	9 of 9	2	
Golden Top Notch (C)	8 of 10	2	
Dark Red Kidney (A)	4 of 10	1	
London Horticultural (A)	3 of 4	3	
McCaslan (A)	8 of 10	3	
Blue Lake Stringless (A)	7 of 17	2	
Kentucky Wonder (A)	13 of 17	1	
do (C)	11 of 19	1	
Kentucky Wonder Wax (A)	11 of 20	1	
Kentucky Wonder Rust Resistant (C)	14 of 20	1	
Alabama Pole No. 1 (C)	10 of 20	1	
Black Stringless Creaseback (C)	10 of 10	2	
Ideal Market (A)	14 of 31	2	10 of 31[c]
Tennessee Wonder (A)	6 of 20	1	20 of 30
Potomac (A)	4 of 12	1	4 of 12
Decatur (A)	4 of 10	2	5 of 10
White Creaseback (C)	20 of 30	2	18 of 30
Genuine Cornfield (A)			21 of 32[c]
Great Northern (A)			12 of 21
U. S. Refugee No. 5 (A)			6 of 18[d]
do (C)			4 of 31[d]
Idaho Refugee (C)			3 of 18
Yard Long (A)			7 of 21

[a] (A) refers to the Asgrow Seed Company; (C) refers to the Corneli Seed Company.

[b] Rating 1, chlorotic or mild necrotic stipple; 2, marked necrotic stipple; 3, leaf necrosis and abscission.

[c] Reddish-brown rings at the infection points.

[d] A few of these plants developed only vienal necrosis of petiole and stem.

Twelve inoculated Lima bean plants, *Phaseolus lunatus* L. var. Baby Potato, developed a few reddish-brown, irregularly outlined, necrotic local lesions, with no systemic symptoms evident. Later three varieties of Lima beans furnished by Corneli Seed Company were also inoculated. Early Bush Market developed numerous local lesions similar to those on the Baby Potato variety. Necrosis spread rapidly along the leaf veins, severely stunting the plants. In Dixie White, small yellow, later necrotic, spots spread along the veins to form irregular brown necrotic areas. The entire leaf soon yellowed, and the plants died within a week or two. Henderson Bush Lima beans developed symptoms similar to, but not nearly so severe as, those on Early Bush Market. The incubation period for all varieties was about 7 days.

Nine of ten inoculated sesame plants, *Sesamum orientale* L., developed within 5 days large necrotic local lesions which rapidly spread throughout the leaf; meanwhile systemic necrosis began to develop in the top of the plant. Five days after local lesions appeared, all of the plants were dead. These results were confirmed by further inoculations.

Petunia, *Petunia hybrida* Vilm. vars. Balcony and Rose of Heaven, developed local and systemic symptoms. The former consisted of small necrotic rings, while the latter were necrotic rings and lines with a severe distortion of the leaves. The virus was recovered from both locally and systemically infected tissue.

Tobacco, *Nicotiana glutinosa* L., did not develop any apparent symptoms, even though numerous inoculations were made; however, sub-inoculations to cowpea var. Black Eye, resulted in 5 lesions on 20 leaves inoculated with sap from symptomless *N. glutinosa* leaves which had developed subsequent to inoculation.

Husk tomato, *Physalis pubescens* L., developed small necrotic rings on the inoculated leaves. No systemic symptoms were observed; however, the virus could be recovered as readily from the young leaves as from the inoculated leaves.

Inoculated green mung bean, *Phaseolus aureus* Roxb., developed no local lesions, but produced a necrosis of the young leaves and stem tips. Only a small percentage of the inoculated plants were infected.

The symptoms on soybean, *Soja max* (L.) Piper, variety S-100, were a systemic stipple necrosis of the young leaves followed by death of the stem tip. Only about 25 per cent of the inoculated plants were infected.

Pea, *Pisum sativum* L. var. Alaska, developed no local lesions but there was a light chlorotic mosaic of the interveinal areas in stunted leaflets developing about two weeks after inoculation.

NON-SUSCEPTS

The following plants did not become infected when inoculated with the guar virus. Sub-inoculations with negative results were made from those species marked with an asterisk. *Leguminosae:* peanut, *Arachis hypogaea*

L.; sesbania, *Sesbania macrocarpa* Muhl.; urd bean, *Phaseolus mungo* L.; adzuka bean, *Phaseolus angularis* (Wild.) W. F. Wright; hairy vetch, *Vicia villosa* Roth.; and crotalaria, *Crotalaria intermedia* Kotschy. *Solanaceae:* pepper, *Capsicum frutescens* L. var. Ruby King*; Jimson weed, *Datura meteloides* L.*; and tomato, *Lycopersicon esculentum* Mill. var. Bonny Best. *Compositae:* zinnia, *Zinnia elegans* Jacq. vars. Giant* and Cut and Come Again; and sunflower, *Helianthus annuus* L. *Amaranthaceae:* globe amaranth, *Gomphrena globosa* L.*

PHYSICAL PROPERTIES

Approximately 18 Giant Stringless Green Pod bean plants were inoculated with each of 12 dilutions of the virus sap between 1:1 and 1:1,000,000. The greatest dilution at which infection occurred was 1:1000. Similar results were obtained in two other tests in which a local lesion host, Black Eye cowpea, was used. In all tests there was a proportionate decrease in infection with dilutions from 1:30 to 1:1000.

Expressed plant sap was put into small test tubes attached to thermometers. Then they were immersed in water which was kept at the given temperature for 10 min. The heating action was checked by plunging the tubes into cool water. In the first test the temperatures used were at 5-degree intervals between 40° and 70° and at 80°, 90°, and 95° C. Approximately 30 Giant Stringless Green Pod bean plants were inoculated with sap from each treatment. Infectivity began to decrease with virus heated to 55° C. A single plant was infected with virus heated to 65° and none with virus heated to 70° C. In the second test the temperatures used were at 2-degree intervals between 57° and 67° C. Approximately 40 primary leaves of Black Eye cowpea were inoculated with juice from each treatment. The average numbers of lesions per leaf were as follows: Check, 19.0 lesions; 57°, 25.9; 59°, 16.3; 61°, 20.8; 63°, 14.6; 65°, 5.2; and 67°, 1.2. Apparently the thermal inactivation point is between 67° and 70° C.

Thoroughly macerated tissue of infected guar plants, with enough water added to make a thin paste, was packed into large test tubes and stored in the refrigerator, some as a chilled liquid and some frozen in the freezing compartment. Under both conditions, the material stored was found to be somewhat infective after 2 months but not after 4 months.

Infected Giant Stringless Green Pod bean leaves were found to lose their infectivity upon drying to a crisp. The extract was made by wetting the leaves with water and macerating them.

The rapid spread of the disease in the guar planting suggests that an insect vector is responsible for its rapid dissemination. In studies of virus transmission through guar seed, 2200 seedlings from seed of infected plants were observed for 35 days, at which time 0.2 per cent of the plants had symptoms similar to those produced by the virus.

COMPARISON WITH SOME KNOWN VIRUS DISEASES

Guar virus symptoms on certain bean varieties are similar to those described by Zaumeyer and Harter (18) for bean mosaic viruses 4 and 4A. However, other varieties differentiate between these and the guar virus. Likewise the guar virus infects cowpea while the bean viruses do not. The thermal inactivation point for the bean viruses is 95° C. compared to 68° C. for the guar virus.

Holmes (3) recognizes 7 strains of cucumber mosaic virus. Six of these produce dark red necrotic lesions on Black Eye cowpea (9) that are similar to those caused by the guar virus. However, 5 of these, *Marmor cucumeris* vars. *vulgare, commelinae, phaseoli, lilii,* and *judicus* Holmes, infect zinnia and may be recovered from it by sub-inoculation (10), while the guar virus failed to infect zinnia. The 6th strain, *M. cucumeris* var. *upsilon,* is inactivated by exposure at 52° C. for 10 min.

The "S" strain of potato virus "X" (*Marmor dubium* var. *annulus* Holmes) produces ringspot symptoms on tobacco (12). However, pepper is highly susceptible to this potato virus but was not infected with the guar virus in repeated inoculations.

A virus disease has been observed on commercial bean varieties in Louisiana and Mississippi with symptoms on several hosts and certain physical properties that are very similar to those of the guar virus (5).

The similarities between the symptoms of the guar virus on bean and tobacco and those reported for tobacco ringspot were such that direct comparisons were considered necessary. To make this possible, Dr. W. C. Price kindly furnished inoculum of the tobacco ringspot virus described by Wingard (17). In comparative inoculations of the two viruses on guar, there was no development of systemic lesions on the young leaves of the plant inoculated with the tobacco ringspot, although stem necrosis began at the point of petiole attachment of the inoculated leaf.

Comparative inoculations of sesame with the two viruses resulted in very similar symptoms, both being rapidly lethal. Likewise, Black Eye cowpea receiving comparative inoculations developed very similar symptoms from the two viruses, the local lesions caused by the guar virus being slightly larger.

Comparative inoculations were made on vigorous Turkish tobacco plants growing in a floor bed of the greenhouse. The local lesions on the plants inoculated with the guar virus consisted of very narrow necrotic rings, with many very fine concentric secondary lines forming about the initial ring. This gave the entire lesion a diffuse appearance. The lines were much wider and with fewer secondary rings on the plants inoculated with the ringspot virus. There were few qualitative differences between the systemic symptoms of the two viruses (Fig. 2). The systemic necrotic rings tended to develop on the guar virus infected plants for a longer period, and the color intensity of the recovered leaves was less than in

leaves similarly infected with the tobacco ringspot virus. The mean bi-hourly temperature in the greenhouse was 80.6° F. for a 20-day period following inoculation.

Valleau (14) has stated that plants systemically infected with tobacco ringspot produce a reduced amount of pollen and that a large percentage of the pollen grains are smaller than normal. Later (15) he found this to be true for 6 different strains of the virus. Representative samples of pollen from plants systemically infected with each of the two viruses were mounted in lacto-phenol with dilute acid-fuchsin as a stain. Measurements were made with an oil immersion objective and ocular micrometer with unit spaces equivalent to 1.37 microns. The number of plasmolyzed grains in each field was recorded. The results (Table 2) show a close similarity between the two groups.

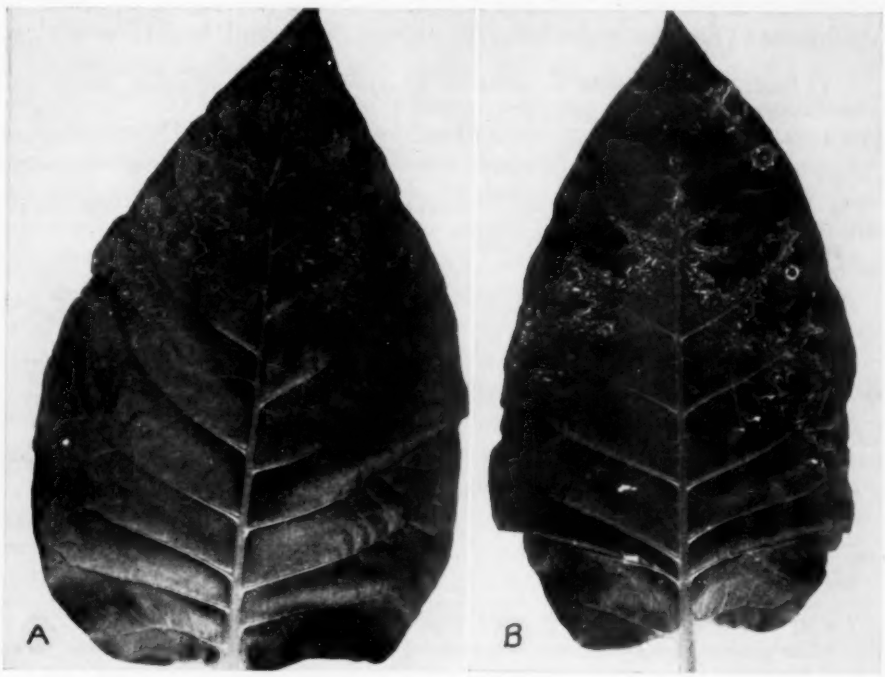

FIG. 2. Comparison of systemic symptoms of guar virus (A) and tobacco ringspot virus (B) on Turkish tobacco. Note the differences in shading of the areas inside and outside of the oak-leaf outline, and in the thickness of the necrotic lines. (A × ½, B × ⅓)

Considering the different viruses producing ringspot symptoms on tobacco, the following characteristics will differentiate between them and the guar virus. The Bergerac ringspot virus, *Annulus bergerac* H., is, in addition to differences of symptoms, inactivated at 80° C. in 10 min. (13, pp. 285–289). Tobacco streak-disease virus, *A. orae* H., also differs in several respects, the greatest being that it is inactivated at 53° C. (4). Tomato ringspot, *A. zonatus* H., is inactivated by temperatures of 55–60° C. The guar virus did not infect tomato.

Three strains of tobacco ringspot are recognized by Holmes (3). The typical strain, *A. tabaci* var. *virginiensis* H., produces local lesions on bean, is more severe on tobacco, and is not typically systemic on guar. The yellow strain, *A. tabaci* var. *auratus* H., causes a definite chlorosis of systemically infected tobacco plants (14). The green strain, *A. tabaci* var. *kentuckiensis* H., is not highly lethal to cowpea when compared to the typical strain (11), whereas the guar virus was just as lethal as the typical strain of tobacco ringspot.

The author had thought that perhaps the guar virus, the soybean bud blight virus, and the bean virus studied by LeBeau were all a variant of tobacco ringspot virus that was highly infective to legumes. However, Allington (1) recently demonstrated the soybean bud blight virus to be

TABLE 2.—*Comparative measurement of Nicotiana tabacum pollen grains from plants systemically infected with tobacco ringspot and guar viruses*[1]

Diameter (microns)	Ringspot Virus		Guar Virus	
	Frequency	Percentage	Frequency	Percentage
Plasmolyzed[2]	71		64	
20.55	1	0.38	4	0.51
21.92	5	1.88	7	2.64
23.29	7	2.63	18	6.79
24.66	22	8.27	35	13.21
26.03	34	12.73	42	15.85
27.40	77	28.95	53	20.00
28.77	83	31.20	53	20.00
30.14	33	12.48	35	13.21
31.51	4	1.50	11	4.15
32.88			3	1.13
34.25			2	0.75
35.62			1	0.38
36.99			1	0.38
Total	266	100.02	265	100.00

[1] Measurements made May 13, 1945, from greenhouse grown plants.
[2] The plasmolyzed grains were not included in calculation of percentages and totals.

identical with the typical strain of tobacco ringspot virus when compared on tobacco, Red Kidney bean, and cucumber (*Cucumis sativus* L.).

It is concluded that host ranges, symptoms, and physical properties of the guar virus and the tobacco ringspot virus show a relationship. However, since certain differences do exist, the guar virus is considered to be a new and distinct variety of tobacco ringspot virus, for which the the name *Annulus tabaci* Holmes var. *cyamopsidis* is proposed.

SUMMARY

An undescribed virus was found naturally infecting guar, *Cyamopsis psoraloides* DC. The infections increased in the field from less than 0.1 per cent to more than 90 per cent in 2½ months.

The virus was sap-transmissible to bean, Lima bean, soybean, cowpea,

green mung bean, tobacco, *Nicotiana glutinosa* L., husk tomato, Jimson weed, garden pea, and sesame. It failed to infect peanut, sesbania, urd bean, adzuka bean, hairy vetch, crotalaria, pepper, *Datura meteloides* L., tomato, zinnia, sunflower, and globe amaranth.

It was systemically infective to 42 varieties of bean, 5 of which also showed local lesions and veinal necrosis. Six varieties developed only local lesions and veinal necrosis. On 6 varieties of cowpeas the virus produced local lesions from which necrosis progressively spread, becoming lethal in 5 of them.

The thermal inactivation point was approximately 68° C. for 10 min. exposure. The dilution end point was 1:1000 and the virus was inactivated in air dried tissue. Macerated infected tissue retained some of its infectivity for 2 months when kept refrigerated at 5° C. or frozen. It was non-infective after 4 months storage.

Comparative studies indicated that pollen grains developed in completely infected tobacco plants were reduced in size and uniformity by the guar virus as well as the tobacco ringspot virus. A very small percentage of seed from infected guar plants apparently transmitted the guar virus.

There are many points of similarity between the typical tobacco ringspot virus and the guar virus. However, the following differences do exist: 37 of 48 varieties of bean failed to produce local lesions as is typical of tobacco ringspot. The guar virus did not infect zinnia and its symptoms are milder on tobacco than those of the typical strain of tobacco ringspot virus. The latter also produced different symptoms on guar.

A comparison of host ranges, the symptoms on various suscepts, and the physical characteristics of tobacco ringspot virus and the guar virus shows a close relationship. However, the differences are believed to be such as to warrant considering the guar virus a new and distinct variety of the tobacco ringspot virus, for which the name *Annulus tabaci* Holmes var. *cyamopsidis* is proposed.

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CULTURAL VARIATION, TAXONOMY AND PATHOGENICITY OF FUSARIUM SPECIES ASSOCIATED WITH CEREAL ROOT ROTS[1]

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INTRODUCTION

To determine the causal fungi associated with cereal root rots[3] in California, and their relative frequency, numerous isolations were made over a four-year period. A large proportion proved to be Fusaria. Because of their evident importance in the root rot complex, an investigation of the variation, pathogenicity, and taxonomic relationships of the Fusarium isolates was undertaken.

Species of *Fusarium* in Wollenweber and Reinking's (21) sections Discolor, Roseum, and Gibbosum were encountered primarily and attention was focused upon these. In these sections according to Dickson (4), pp. 207–209, only three species are generally considered to be of primary importance as cereal root rot pathogens, *Fusarium graminearum* Schw. and *Fusarium culmorum* (W. G. Sm.) Sacc. of the section Discolor and *Fusarium avenaceum* (Fr.) Sacc. of the section Roseum. In the section Gibbosum, Bennett (2) and Johnston and Greaney (12) have demonstrated *Fusarium equiseti* (Cda.) Sacc. to be weakly pathogenic, as have Gordon and Sprague (7), Sprague (18), and Bennett (1) for certain varieties and strains of *Fusarium scirpi* Lamb. et Fautr.

Cultural variation is a common phenomenon in the cereal Fusaria. Eide (5), Goddard (6), Ullstrup (20), and Tu (19) have reported variants in *F. graminearum*. Tu (19) likewise demonstrated variants in *F. culmorum* and *F. avenaceum*. Variation in *F. culmorum* and *F. sambucinum* was shown by Mitter (13).

That variation in pathogenicity accompanies cultural variation has been shown by Ullstrup (20), Eide (5), and Tu (19). All have demonstrated cultural variants to be less pathogenic than the parental type. Johnston and Greaney (12) found, in nature, isolates of *F. culmorum* ranging from severe parasites to practical saprophytes, some even less pathogenic than *F. equiseti* and *F. avenaceum*.

Positive identification of species and forms within the genus *Fusarium* has been difficult and usually is undertaken only by those having special

[1] The work herein reported was part of a thesis presented to the Graduate Division of the University of California, May, 1942, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The writer wishes to acknowledge his indebtedness to Dr. W. C. Snyder and Dr. H. N. Hansen for their advice and criticism throughout these investigations.

[3] In this paper root rot is used in its broadest sense to include injury to root, crown, basal culm, and leaf sheath tissue.

knowledge of their characteristics and relationships. The problem of taxonomy is complicated by the numerous cultural variants that appear and by the diversity of forms that occur in nature.

The standard taxonomic system has been that contained in Wollenweber and Reinking's monograph, *Die Fusarien* (21), in which the genus is divided into 16 sections each containing species and forms. Evidence that taxonomic lines drawn by this work have been too fine for practical usage has been brought forth by Hansford (11), Snyder and Hansen (14), Mitter (13), and Brown (3). Some investigators have observed that variants of a given species cross not only species lines but in some cases sectional groupings (13, 17). Snyder and Hansen (14, 16, 17), in pointing out the unreliability of such key characters as conidial size and shape, presence and type of chlamydospores, and stromatal color, have completed an extensive revision of the genus reducing the species to nine. They propose the use of host relationships as bases upon which to separate the pathogenic forms within morphological units. In the cereal Fusaria they have reduced all species, varieties and forms of the sections Discolor, Roseum, and Gibbosum[4], to the species *Fusarium roseum* (Lk.) Sny. and Hans. All forms pathogenic on cereals are denoted as *F. roseum* forma *cerealis*.

The investigations reported here were undertaken (1) to find the range of variability in the *Fusarium* species which had been isolated in pure culture, using the single spore technique throughout; (2) to obtain evidence on the question of reversion of variants to the parental type; (3) to compare the cultural variants obtained in these studies with their parent types, and to determine the species identity of each variant independently of any knowledge of the identity of the parent type from which it came; (4) to compare the pathogenicity of the different species of *Fusarium* isolated and the pathogenicity of the parent types with that of the cultural variants; and (5) to obtain a clearer evaluation of the relationships of the Fusaria that cause root rot of cereals.

CULTURAL STUDIES

Methods.—To initiate each isolate in pure culture, twenty of the first conidia produced on the mycelium advancing from the diseased tissue were isolated singly. When perithecia were available, single ascospore cultures were made directly.

Except where specified, isolates were transferred by employing single conidia as inocula following the method used by Hansen and Smith (9). Large test tube slants of potato-dextrose agar were used as media throughout. All cultures were maintained at room temperatures (23°–25° C.) and exposed continuously to diffuse daylight. Sporulation was more profuse and normal under these conditions, an observation in agreement with that of

[4] Wollenweber and Reinking (21) separate these 3 sections as follows: species in the section Roseum lack chlamydospores; those in the section Gibbosum have intercalary chlamydospores; and those in the section Discolor have intercalary and sometimes terminal chlamydospores.

Snyder and Hansen (15). To avoid the possibility of culture mite contamination, the tubes were capped with cigarette paper as described by

TABLE 1.—*Sources of isolates of Fusarium species, their cultural type, and their relative pathogenicity on wheat seedlings*

Species designation of field isolate	Isolate	Origin	Cultural type[a]	Disease rating[b]
Section Discolor				
F. graminearum	3	wheat	I	2.5
Do	4	wheat	I	4.8
Do	4A	culture variant of 4	II	2.0
Do	5	barley	I	3.1
Do	6	wheat (perithecia)	I	4.3
Do	6A[c]	culture variant of 6	II	0.7
Do	7	wheat	II	0.9
Do	11	corn	I	5.0
Do	13	barley	I	3.9
F. culmorum	1	barley	I	3.7
Do	2	barley	I	3.1
Do	3	barley	I	3.6
Do	3A	culture variant of 3	I	2.1
Do	4	wheat	I	4.0
Do	4A[d]	culture variant of 4	II	0.1
Do	5	corn	I	4.5
Do	5A[e]	culture variant of 5	II	0.1
Do	7	barley	I	2.8
Do	7A[f]	culture variant of 7	II	1.3
Do	7B[g]	culture variant of 7	II	0.8
F. sambucinum	1	barley	I	0.0
Do	2	Stipa sp.	I	0.0
Do	2A	culture variant of 2	II	0.0
Section Gibbosum				
F. equiseti	1	wheat	I	0.5
Do	2	wheat	I	0.7
Do	2A[h]	culture variant of 2	II	0.3
F. scirpi	1	wheat	I	0.0
Do	2	Stipa sp.	I	0.0
Section Roseum				
F. avenaceum	1	peas	I	1.0
Do	1A	culture variant of 1	II	0.4
	Check		.....	0.0

[a] Type I is a culture with abundant aerial mycelium with conidia borne in sporodochia; Type II cultures are appressed with numerous conidia in pionnotes.

[b] Three replications of 15 wheat plants each, variety Baart 38, were used to test each isolate. The plants were grouped into 6 classes from 0 to 5 increasing with severity of the disease (0 = healthy; 5 = dead), and a disease rating was calculated for each replicate. Significant differences between average disease ratings; 5 per cent level, 0.7: 1 per cent level, 1.0.

[c] Variant 6A reclassified as *Fusarium sublunatum* var. *elongatum* Rg.

[d] Variant 4A reclassified as *Fusarium flocciferum* Corda.

[e] Variant 5A reclassified as *Fusarium reticulatum* Mont. f.1 Wr.

[f] Variant 7A reclassified as *F. culmorum* var. *cereale* (Cke.) Wr.

[g] Variant 7B reclassified as *F. sambucinum*.

[h] Variant 2A could not be fitted into existing classification.

Hansen and Snyder (10). Spore measurements were made under high power of the microscope with a filar micrometer.

Sources of isolates.—In table 1 the isolates and variants selected for these investigations are listed together with their origin, cultural type, and disease rating. As each isolate was obtained from nature it was designated with the binomial in Wollenweber and Reinking's *Die Fusarien* (21) that appeared to fit it most accurately. No isolate having the characteristics of the section Roseum was obtained from California cereals. Some isolates originally placed in this section later had to be considered as belonging to Gibbosum. A culture from pea roots from Hamilton, Montana, was used to represent this group. The isolates studied include three species of the section Discolor, two of the section Gibbosum, and one of the section Roseum.

Cultural type of field isolates.—From the initial single-spore isolations from naturally diseased tissue two types of cultures resulted, an expression of the Hansen (8) dual phenomenon. Throughout the rest of the studies these two were evident in all six Fusarium species investigated. They will be referred to as Types I and II hereafter.

Type I is characterized by an abundance of aerial mycelium often filling the culture tube. The conidia are ordinarily produced in sporodochia but occasionally are found sparsely scattered in groups in the aerial mycelium.

Type II has little or no aerial mycelium, the organism growing closely appressed to and in the agar. As cultures of this type age, they usually take on a slimy appearance. Conidia are produced in great abundance in pionnotes or sometimes in pseudopionnotes.

The letters C and M as used by Hansen (8) to designate the two cultural types of fungi are not used as they are not applicable to the cereal Fusaria. In Hansen's work with Fusaria of the sections Elegans and Martiella, the primary type was conidial (C) and the secondary, mycelial (M). In the cereal Fusaria the primary culture type (I) has abundant aerial mycelium and the secondary form (II) is pionnotal rather than mycelial. Type I appears to correspond to Eide's (5) Types A-H and Ullstrup's (20) Type A, and Type II to Eide's Type I and Ullstrup's Type B as described in their respective studies with *F. graminearum*.

Of 390 conidia of 5 species of *Fusarium* isolated singly from nature, 365 were of culture Type I. In the instance of isolate 7 of *F. graminearum* all 20 initial single conidia produced colonies of the pionnotal Type II. This indicates the organism probably occurred in nature in that form. An isolate of *F. sambucinum* yielded cultures of both types in equal numbers. Perithecia of *Gibberella zeae* (Schw.) Petch were available in one case and 40 single ascospores produced colonies exclusively of Type I. The results are in accord with those of Eide (5) and demonstrate that although the sporodochial Type I is predominant, the pionnotal Type II does appear to occur in nature. Furthermore, a similar situation was met with in *F. culmorum*, *F. scirpi*, *F. equiseti*, and *F. sambucinum*.

Variation in culture.—Twenty-seven different isolates, comprising six species of *Fusarium*, were analyzed through 3 to 4 generations by employing the following single-spore method: The original single-spore isolates were

kept at least 8 weeks, and in most cases over a year, before the second single-spore generation was initiated in order to allow time for maximum variation in the original single-spore cultures. At least 10 single spores were isolated from the original single-spore culture. If all of the cultures in the second generation were identical as to cultural characters only one was carried into the third generation. If, on the other hand, variants occurred along with the parental type, both were continued into the next generation. Each culture was carried through at least three generations by this technique.

Of the approximately 600 single-spore transfers of isolates representing the six species of *Fusarium*, the resulting cultures always could be placed with ease into either cultural Type I or Type II. There was variation within the Type I and Type II cultures as to color, amount of mycelium and

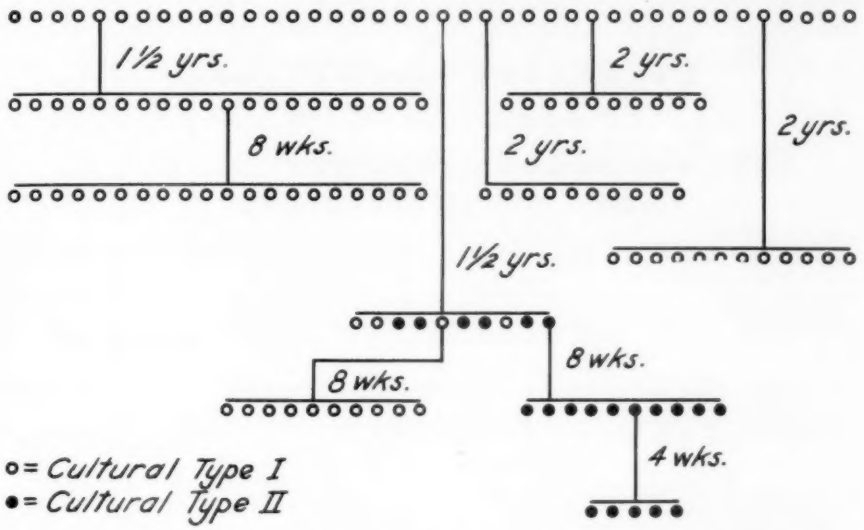

FIG. 1. Single-spore analysis of an isolate of *F. graminearum* (*Gibberella zeae*). Each circle in the first generation represents a single-ascospore culture direct from nature. Thereafter each circle represents a single-conidium culture. Time intervals between transfers are indicated along lines connecting generations. Type I refers to the sporodochial type most commonly isolated from nature and II to the pionnotal variant. (See fig. 3.) Of the 5 ascospore cultures carried into the second generation, one mutated to the pionnotal type and remained constant thereafter.

sporulation, but there was always a definite distinction between the two general types.

In each of the six species there were isolates that gave rise to Type II pionnotal variants during the course of single-spore transfer. Three of 13 isolates of *F. graminearum*, and 3 of 7 of *F. culmorum* yielded cultural variants of Type II. In each *F. sambucinum*, *F. scirpi*, *F. equiseti*, and *F. avenaceum* variants of this type were encountered.

A typical analysis of a culture of *F. graminearum* is illustrated in figure 1. Of the five single-ascospore cultures of *Gibberella zeae* carried into the second generation one yielded colonies of the Type II variant as well as the

parent type. This variant remained true to type through two subsequent single-spore generations.

Figure 2 diagrams the cultural behavior of an unstable isolate of *F. equiseti*. After 16 weeks in culture, one of the original single-spore isolates gave rise to 18 Type II variants among the 20 single-spore transfers. These remained constant thereafter. The parental type always yielded a large percentage of variants if sufficient time elapsed between generations. Figure 2 indicates that a period of over 3 weeks was necessary for this variation to occur under ordinary laboratory light and temperature conditions.

There appears to be a general directional change in the cereal Fusaria from the primary Type I, most commonly recovered in nature, to the sec-

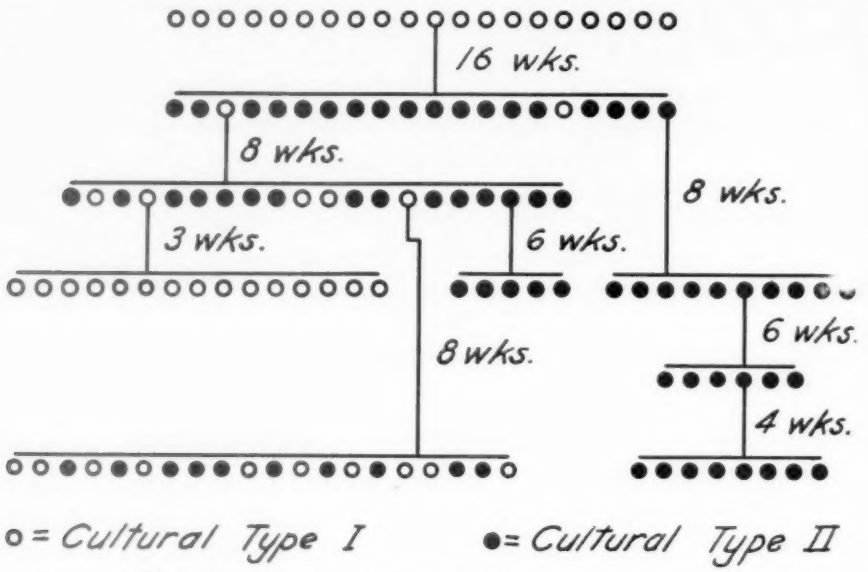

FIG. 2. Single-spore analysis of an isolate of *Fusarium equiseti*. Each circle represents a single-conidium culture, the top row being the original field isolates. Time intervals between transfers are indicated. Type I refers to the sporodochial type as isolated direct from nature and II to the pionnotal variant. In this case Type I is very unstable, continually producing variants of the pionnotal type, all of which remained constant. The effect of time elapsed between transfers is shown in the Type I culture transferred at the age of 3 weeks when no pionnotal types appeared, and at 8 weeks when one-half of its spores were the pionnotal mutant.

ondary Type II, as the organism is propagated in culture. This tendency has been observed by others. Ullstrup (20), in studying two isolates of *F. graminearum* by the single-spore method, showed this change, yet the original types were retained as was the case here. Also isolates of the same species differ in the frequency with which they yield Type II variants.

Figure 3 illustrates cultures of *F. graminearum* and *F. culmorum* as originally isolated, together with their corresponding Type II variants.

Constancy of the pionnotal variants in culture.—Controversy exists in the literature concerning the question of reversion of cultural variants to

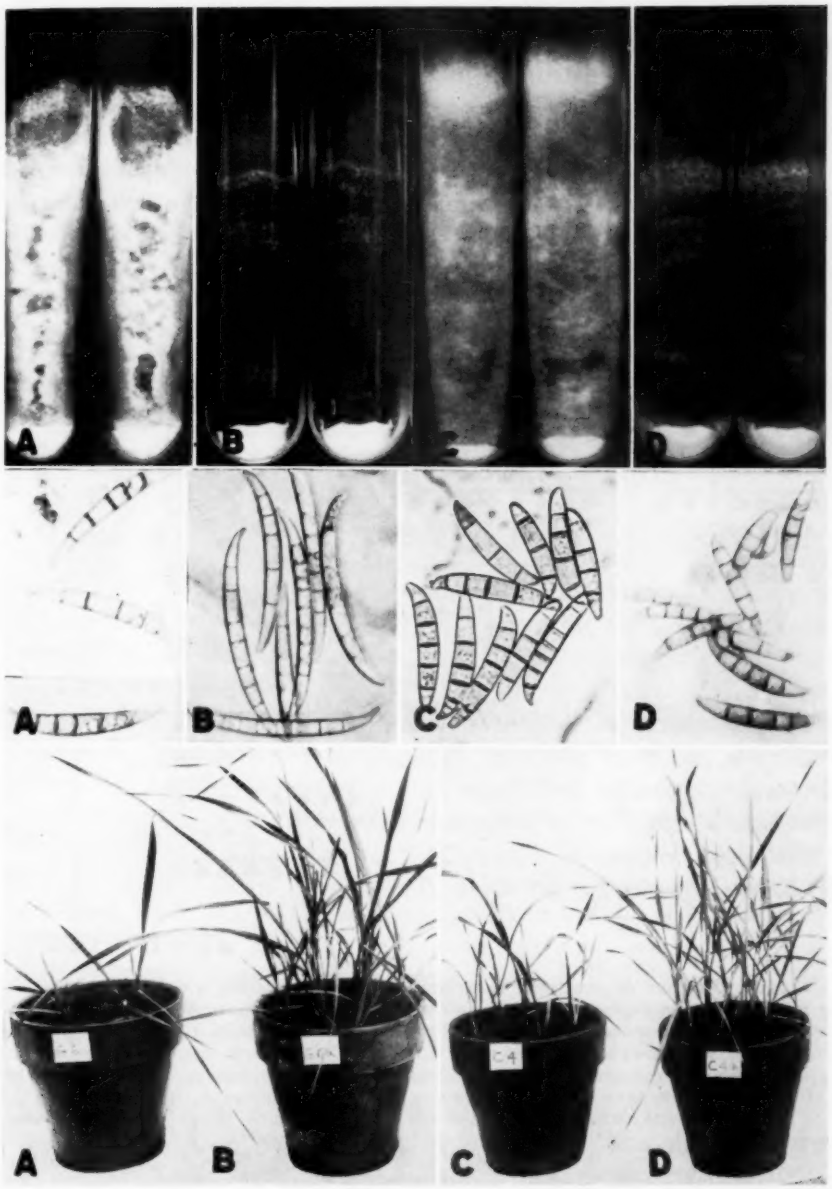

FIG. 3. Comparison of parent and variant single-spore isolates of *F. graminearum* and *F. culmorum* on cultural, morphological and pathogenic bases. A. Parent culture 6 of *F. graminearum*. B. Variant 6A of A reclassified as *F. sublunatum* var. *elongatum*. C. Parent culture 4 of *F. culmorum*. D. Variant 4A of C reclassified as *F. flocciferum*. The top row shows test-tube cultures of the two parents and their variants. Each parent is of the sporodochial Type I, whereas the variants are of the pionnotal Type II. The second row illustrates macroconidia (×450) of each culture. Upon these differences in size of the conidia and other features the variants were no longer considered as the same species as their respective parents. The bottom row shows the marked virulence on wheat seedlings of the two parent types as compared to their pionnotal variants.

the parental types. Working with the same fungus, *F. graminearum*, Goddard (6) reported reversion while Eide (5) and Ullstrup (20) found no evidence of it. Ullstrup (20) demonstrated by the use of the single-spore method of transfer that the pionnotal variant remained constant through ten generations.

In this work the writer tested 12 pionnotal variants of five different species. Five Type II variants of *F. graminearum*, 4 of *F. culmorum*, and one each of *F. equiseti*, *F. sambucinum*, and *F. avenaceum* were carried through 1 to 4 generations by single-spore transfer over a period of

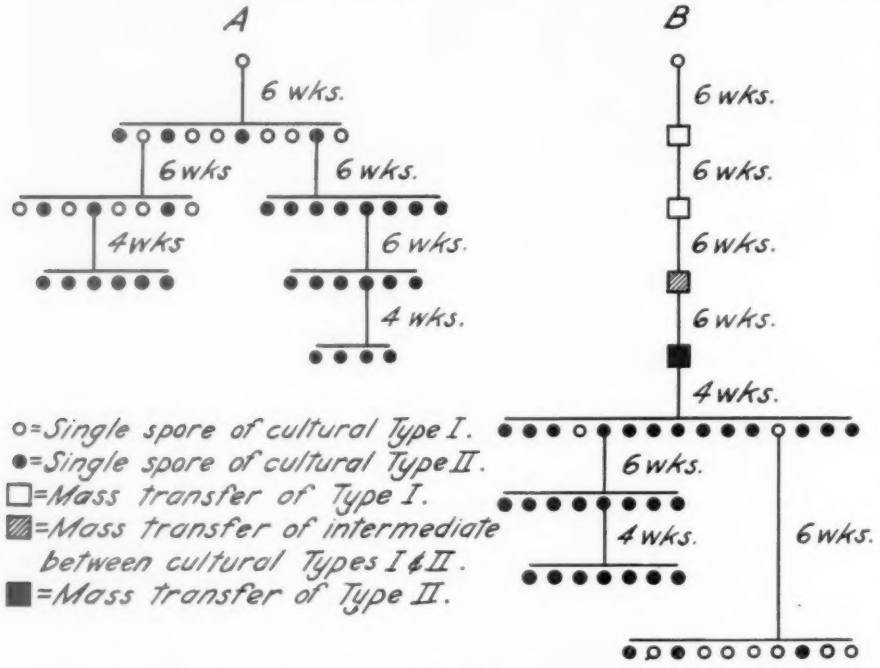

FIG. 4. Diagram of two methods of artificially perpetuating a culture of *F. equiseti*. The two top circles represent single-conidium cultures of the original field isolate. In procedure A, single-spore transfers were made throughout. In B, the original single-spore culture was mass transferred four times and the fourth mass transfer was then single-spored. Type I refers to the sporodochial type from nature and II to the pionnotal variant. Note that by the single-spore method, procedure A, no reversion of Type II to Type I occurs, whereas by mass transfer procedure B, an apparent reversion appears.

years. Of 278 single conidia isolated from Type II cultures, all resulting colonies were exclusively of Type II.

The apparent disagreement seems to be rooted in the methods employed in the various investigations. Goddard (6), using mass transfer methods, obtained reversion, whereas Ullstrup (20) and the writer, employing single-spore subculturing exclusively, obtained no evidence for it. An experiment was devised to compare the method of mass transfer with that of the single-spore technique using the same isolate. The procedures are diagrammed in figure 4.

A Type I culture of *F. equiseti* was selected as it very readily produced a Type II pionnotal variant in culture. Two identical single-spore cultures of Type I from the original field isolate were selected. One was subjected to procedure A, the single-spore method of transferring. In the first generation, four of the ten cultures were of Type II. Three subsequent single-spore generations of this Type II showed it to be constant with no evidence of reversion to Type I. In procedure B, the original single-spore culture was mass transferred four generations in succession. That is, a mass of mycelium was taken at the end of six weeks from the original single-spore culture and transferred to fresh medium. At six-week intervals this was repeated as illustrated in figure 4. The culture resulting from the third mass transfer was materially different from the original type. It had scattered tufts of aerial mycelium, but in general it was of Type II. At this stage the culture obviously consisted of a mixture of Types I and II. The culture after the fourth transfer was wholly Type II in appearance and was identical to the Type II cultures produced in procedure A from single spores. Yet upon isolating 15 single conidia from it, two colonies identical to the original Type I appeared. This lends evidence that real reversion does not occur when the variant cultures are always initiated with single spores; yet when mass transfers are used a false reversion can occur which is probably due to a reassortment of nuclei perpetuated from the outset.

Comparison of the variants with the parent cultures from a taxonomic standpoint.—It has been the tendency of workers to consider a cultural variant as a member of the parent species without attempting to fit it into the taxonomic scheme independently of the parent. Mitter (13) pointed out that certain variants of *Fusarium culmorum* had conidia that differed so much from those of the parent in regard to shape that they could not be considered as belonging in the same species or even in the same section as the parent.

The object of this study was to compare the pionnotal variants (Type II) with parental types (Type I) to see whether or not the parent and its variant, when classified independently of one another according to the criteria set up by Wollenweber and Reinking (21), as was done in section Elegans by Snyder and Hansen (14), would fall into the same species. This was done with representatives of four species. The characters used by Wollenweber and Reinking (21), such as the presence of chlamydospores, spore size and septation, and stromatal color, were studied. It was found that chlamydospores in some species occurred more commonly in water culture than on potato-dextrose agar, so both of these methods were employed.

Table 2 summarizes the results of this comparison. In the last column the variants are given the species names, the description of which most closely fits them in *Die Fusarien* (21).

TABLE 2.—*Comparison of Fusarium variants with their parent cultures from a taxonomic and pathogenic standpoint*

Species and Isolate		Cultural Type[a]	Average spore size (5 septate)	Average spore septation	Chlamydospores[b]		Disease rating[c]	Identification of parent and variant cultures[d]
					On potato dextrose agar	In water culture		
Section Discolor								
F. graminearum	6	I	51.7 × 5.3 μ	5	0	0	4.3	*F. graminearum*
Variant	6A	II	62.4 × 4.9 μ	7	++	++++	0.7	*F. sublunatum* var. *elongatum*
F. culmorum	4	I	40.4 × 6.6 μ	5	+	++++	4.0	*F. culmorum*
Variant	4A	II	35.9 × 4.5 μ	5	+++	++++	0.1	*F. flocciferum*
F. culmorum	7	I	41.0 × 6.7 μ	5	+	+++	2.8	*F. culmorum*
Variant	7A	II	41.5 × 5.7 μ	5	+	+++	1.3	*F. culmorum* var. *cereale*
Variant	7B	II	38.4 × 4.9 μ	5	++	++	0.8	*F. sambucinum*
F. culmorum	5	I	36.2 × 6.6 μ	5	++	+++	4.5	*F. culmorum*
Variant	5A	II	(3 septate) 28.4 × 3.5 μ	3	++++	++++	0.1	*F. reticulatum* f.1
Section Gibbosum								
F. equiseti	2	I	41.9 × 3.8 μ	3	0	+++	0.7	*F. equiseti*
Variant	2A	II	56.2 × 3.8 μ	5	0	++	0.3	new species[e]
F. scirpi	1	I	45.0 × 5.0 μ	5	++++	++++	0.0	*F. scirpi*
Variant	1A	I	No conidia	—	0	0	0.0	new species[f]
Check							0.0	

[a] I represents sporodochial type usually isolated from nature; II represents pionnotal variant.

[b] Number of plus signs indicate relative abundance of chlamydospores.

[c] Ranging from 0 to 5 with increasing severity of disease as measured on wheat seedlings. Difference required for significance at 5 per cent level, 0.7—at 1 per cent level, 1.0.

[d] Identification was made by use of the system of Wollenweber and Reinking (21).

[e] This variant could not be placed in the existing system and a new species or variety would have to be created.

[f] This fungus, due to its absence of chlamydospores, can no longer be placed in *F. scirpi* nor even in the section Gibbosum; it must be considered as being in the section Roseum.

F. graminearum isolate 6, representing the parent Type I, fitted the species description both as to size of conidia and absence of chlamydospores. The Type II variant 6A, however, had much longer and thinner spores with an average septation of 7. In figure 3 the difference between the macroconidia of the parent and variant is shown. Furthermore, the variant contained chlamydospores, which were extremely abundant especially when the culture was grown in water. The length of the macroconidia and the abundance of chlamydospores forbids its classification as *F. graminearum*. Rather it seems to fit *F. sublunatum* var. *elongatum*.

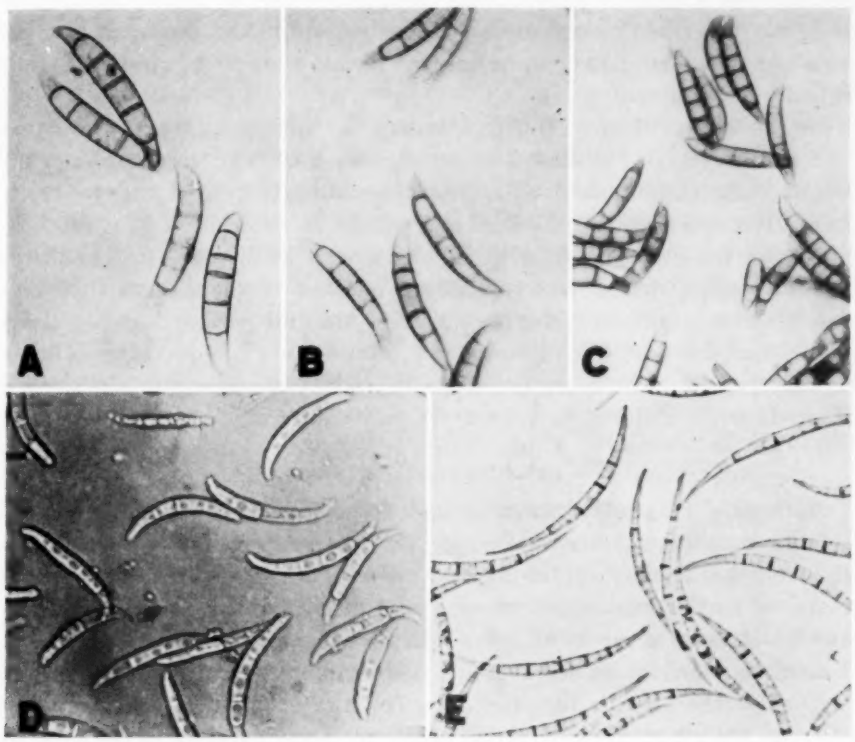

FIG. 5. Comparison of macroconidia of parent and variant isolates of two Fusarium species. A, Macroconidia of *F. culmorum* parent culture 7; B, macroconidia of one of its variants, 7A, reclassified as *F. culmorum* var. *cereale;* and C, macroconidia of its other variant, 7B, reclassified as *F. sambucinum*. D, Macroconidia of *F. equiseti* parent culture 2; and E, macroconidia of its variant, 2A, for which there is apparently no existing species in the section Gibbosum (×450). This difference in the macroconidia of the parent and variant type is one of the factors that necessitates the reclassification of the variants, if the system of Wollenweber and Reinking is to be adhered to closely.

In the three *F. culmorum* isolates studied, four Type II variants appeared, two from one parent culture. While the three parent cultures all fitted the species description, the variants fell into four different places in the taxonomic scheme. One fitted into *F. flocciferum*, another into *F. reticulatum*, and of the two from the same parent, one was *F. culmorum* var. *cereale* and the other, *F. sambucinum*. The variants differed from *F.*

culmorum primarily in their spore width. Figures 3 and 5 illustrate these differences in the macroconidia.

Isolate 2 of *F. equiseti* was originally considered as a member of section Roseum because on potato-dextrose agar no chlamydospores were observed. However, when the fungus was allowed to grow in a water blank for a week, the chlamydospores were produced in abundance. Therefore, it was placed in the section Gibbosum and identified as *F. equiseti*. The Type II variant, 2A, likewise produced chlamydospores only in water, but the conidia were markedly different from those of the parent in length and septation, as shown in table 2 and figure 5. There was apparently no previously described species or variety that would fit this particular variant and a new one would have to be created if this were to be classified in the Wollenweber system.

In the study of the *F. scirpi* isolate, a variant appeared which remained in Type I but had less aerial mycelium and no conidia. This variant completely lacked chlamydospores and therefore had to be removed from the section Gibbosum and placed in the section Roseum. The parent, on the other hand, with its characteristically curved conidia and knots of resting spores, was typically a member of the section Gibbosum.

A taxonomic difference between the variant and parent does not always occur. Culture 4 of *F. graminearum* produced a pionnotal Type II variant culture, 4A, which on the basis of spore measurement and lack of chlamydospores differed in no way from its parent.

PATHOGENICITY STUDIES

Methods.—To study pathogenicity of the Fusarium isolates and their variants, greenhouse tests were employed. Several preliminary trials were conducted with many of the isolates to determine the best host. Wheat proved to be the most satisfactory, being generally more susceptible than barley. Oats were rarely affected. In these early trials different methods of artificial inoculation were used. The application of heavy spore suspensions to the seed or into the open furrows at planting was successful with the abundantly sporulating isolates. This method, however, could not be used in any trial that included all the isolates, for many sporulated only sparsely or not at all.

Soil inoculation with infested oat kernels was used because a standard amount of inoculum could be employed for all the fungi tested. It was recognized that the amount of inoculum used or inoculum potential could strongly affect the apparent pathogenicities of most of the isolates that showed any degree of parasitism. An attempt was made to discover the optimum inoculum potential, *i.e.*, a standard amount of inoculum that would adequately segregate the severe, moderate, and weak parasites.

Having determined the best host plant and the most satisfactory inoculum potential, a replicated experiment including all the isolates and variants was set up. From a single-spore culture of each isolate, transfers

were made in triplicate to three tubes of moist sterilized oat kernels. Each tube was made up with 15 grams of dry oats and 20 cc. of distilled water.

When infestation of the oats was complete, the contaminated kernels from each tube were mixed in the sterile soil of a 6-in. pot. Sterile oats were similarly added to each check pot. After allowing 3 days for the fungi to become established, fifteen seeds of wheat, variety Baart 38, were planted in each of the 3 pots for each isolate tested.

During the trial the soil temperatures ranged from 22° to 28° C., averaging 25° C. At regular intervals approximately equal quantities of water were added to all pots to maintain a soil moisture level favorable for plant growth.

At the end of 24 days, the plants were dug, washed, and grouped into 6 classes ranging from 0 to 5, according to the severity of the disease. Both top and underground symptoms were taken into account. The 0 represented a healthy vigorous plant with no discoloration of the crown or roots. Class 1 plants were those that were apparently healthy in regard to top symptoms but which had definite brown lesions on the crown. These lesions were only in the leaf sheath, not penetrating through to the culm. Class 2 plants were somewhat stunted, having rather severe rotting and discoloration of the basal leaf sheath, culm, and sub-crown internode. Class 3 represented plants definitely stunted and containing a severe rotting of the crown. Plants that were placed in class 4 were those that barely emerged and were dead, or nearly so, by the time of harvesting. Class 5 represented plants that were killed prior to emergence.

A mean disease rating was calculated for each replicate by multiplying the number of plants in each class by the class index number, summing the products, and dividing by the total number of plants. An analysis of variance was then applied to the average disease ratings of the 30 isolates and the check which showed that a difference in disease rating of 0.7 was required for significance at the 5 per cent level and 1.0 at the 1 per cent level.

Experimental Results.—In preliminary trials with small groups of isolates, great variation in pathogenicity was observed among the isolates of the six Fusarium species. In particular, the variants of cultural Type II showed a much lower order of parasitism than their respective Type I parent cultures. The experiment described above which included all 30 isolates in the same trial substantiated the earlier preliminary investigations. Table 1 presents the disease ratings of the isolates together with their cultural types. Table 2 includes a comparison of parent and variant cultures on a pathogenicity basis.

All isolates and variants of *F. graminearum* proved to be pathogenic, ranging from 5.0 to 0.7 in disease rating. Isolates of cultural Type I had disease ratings of 2.5 and over. On the other hand the cultures of Type II were less virulent, varying from 2.0 to 0.7 in their disease ratings, and

when compared with their parent cultures were significantly less parasitic as shown in figure 3 and table 2. It is of interest to note that the pionnotal variant of 4A of this species was relatively pathogenic with a rating of 2.0, whereas the variant 6A was parasitic only to the extent of 0.7. Taxonomically, variant 4A remained as *F. graminearum* whereas 6A became *F. sublunatum* var. *elongatum*.

As in *F. graminearum* all isolates and variants of *F. culmorum* were pathogenic, but again there was great variation among disease ratings of the cultures, ranging from 4.5 to 0.1. Comparison of the parent Type I cultures with their Type II pionnotal variants again demonstrated the weak pathogenicity of the latter type. (See figure 3 and table 2.) The pionnotal variants which were reclassified taxonomically were all pathogenic to a slight extent as follows: *F. culmorum* var. *cereale*, 1.3; *F. sambucinum*, 0.8; *F. flocciferum*, 0.1; and *F. reticulatum* f.1, 0.1.

Both isolates of *F. equiseti* proved to be slightly pathogenic on wheat seedlings. With this species there was no pre-emergence killing or seedling blight. The disease when present was manifested only as brown lesions on the basal leaf sheaths. The pionnotal variant was slightly pathogenic, but, as in the other species, was less so than the parent form.

Neither isolate of *F. scirpi* showed any evidence of parasitism on wheat seedlings. Strains of *F. scirpi* v. *acuminatum* (Ell. et Ev.) have been proved mildly parasitic by Sprague (18), as have the varieties *F. scirpi* v. *pallens* and *F. scirpi* v. *nigrans* by Bennett (1). The absence of parasitism on seedlings does not rule out the possibility of the cultures herein tested being pathogenic to some degree upon older plants.

Three cultures of *F. sambucinum* obtained from nature likewise caused no disease on wheat seedlings. However, a culture of this species that was obtained as a variant from *F. culmorum*, 7B, showed a low order of pathogenicity, 0.8.

To represent the section Roseum, an isolate of *F. avenaceum* from peas from Montana was tested along with the other species. The pionnotal variant again proved to be less pathogenic than the parent type, having a disease rating of 0.4 as compared to 1.0 for the parent.

Summarizing the data obtained from the pathogenicity studies, the following things become apparent: (1) all isolates of all species except *F. scirpi* and *F. sambucinum* were pathogenic to some extent on wheat seedlings; (2) a culture of *F. sambucinum* that arose as a variant from *F. culmorum* was slightly virulent; (3) the variants of cultural Type II were significantly less parasitic than their parental type in all cases; (4) cultures of *F. sublunatum* var. *elongatum*, *F. flocciferum*, and *F. reticulatum* f.1 were slightly virulent, which constitutes, as far as is known, the first report of their parasitism on cereals; and (5) within one species, *F. graminearum*, was exhibited a range of pathogenicity similar to that of the whole group of species and sections.

DISCUSSION

These investigations with numerous isolates of the cereal Fusaria, sections Discolor, Roseum, and Gibbosum, disclosed that there were two general cultural types into which all could be placed. The first type was that having an abundance of aerial mycelium with conidia borne usually in sporodochia but occasionally directly in the mycelium (Type I). Cultures with appressed mycelium covered with conidia borne in pionnotes constituted the second type (Type II). Though the former was the most common type, evidence was obtained that the latter type did occur in nature.

Each of the six species studied yielded, upon subculturing with single conidia as inocula, variants of Type II, all of which remained stable in subsequent generations of single-spore culture. Only by continual mass transfer of the original types and the subsequent variants was it possible to obtain reversion of the Type II variant to the parent form. Such apparent reversion is interpreted as a segregation of nuclei that were present from the outset and perpetuated by mass transfer.

When these variants were classified without knowledge of their parent type, many of them fell into species different from those of their parents and some even into different sections. Two of the variants could not be placed in the existing system. Such evidence as this weakens the usefulness of the Wollenweber and Reinking (21) system. Segregation of the cereal Fusaria into distinct morphological units or species becomes difficult when a variant of one species falls into another of the previously described species. New variants may be encountered, as was the case in this work, that may not fit into the existing system and will require new binomials. If these display pathogenicity, as many have thus far, the Fusarium root rot of cereals will have to be considered as caused by an ever-increasing number of *Fusarium* species.

Neither can separation of the sections or species within the sections on a basis of degree of pathogenicity be consistently workable. Members of all show parasitism to some degree on wheat seedlings. Within single species such as *Fusarium graminearum* and *F. culmorum* a range of pathogenicity is exhibited similar to that of the entire groups of species. Johnston and Greaney (12) reported field isolates of *F. culmorum* ranging from very virulent to some that were less pathogenic than *F. equiseti* and *F. avenaceum*.

In view of two facts: first, that members of these sections are difficult to distinguish on the basis of their pathogenicity since single species may embrace the range of pathogenicity of the whole group; and second, that variants of one species may fall into other species or even into other sections on a morphological basis, there would appear to be a close relationship between the three sections and the species comprising them.

How then may the plant pathologist consider this aggregate of Fusarium species, varieties, and forms which cause the cereal root rots? In

the literature, reference is often made merely to *Fusarium* spp. causing cereal root rots. Snyder and Hansen (17) have placed all the species, forms, and varieties of these three sections into one species, *Fusarium roseum* (Lk) Sny. and Hans., designating the forma *cerealis* for all isolates proven pathogenic on cereals.

It should be noted that the writer throughout the text refers to the isolates with definite binomials yet suggests that the relationships were so close that a grouping seems warranted. At the outset, for convenience in handling, each field isolate was identified as closely as possible by reference to *Die Fusarien* (21). As they accumulated, the author was able to recognize certain groups of isolates as fitting into particular species. Other isolates seemed to bridge the gaps between species and varieties within the three sections and were arbitrarily given the name of the species which they most closely resembled. Because many of the variants of a recognized species fell into other recognized species, a definite interrelationship was indicated.

SUMMARY

In a study of the cereal root rot complex in California, isolates of Fusaria were commonly encountered. Because of their evident importance in the root-rot disease, an investigation of the variation, pathogenicity, and taxonomic relationships was undertaken.

Each culture was initiated in pure culture by isolating 20 single conidia directly from the first mycelium advancing from diseased host tissue. These isolates were identified by reference to Wollenweber and Reinking's *Die Fusarien*. Some could be keyed with ease to definite species but others bridged the gaps between species and sections and were arbitrarily given the binomial they most closely resembled. Isolates of *F. graminearum*, *F. culmorum*, *F. sambucinum*, *F. equiseti*, *F. scirpi*, and *F. avenaceum*, representing the sections Discolor, Gibbosum, and Roseum, were included in the studies.

All isolates of all species could be placed in one of two major types, I and II, based on their cultural characters. Type I cultures had abundant aerial mycelium, and the conidia were in sporodochia. This was the type usually found in nature. Type II cultures were appressed and slimy due to the production of numerous conidia in pionnotes. In two cases pionnotal type II isolates were obtained directly from nature, but usually they occurred as culture variants.

All isolates were propagated through 3 to 4 generations by the single-spore technique of transfer. Type I cultures when analyzed by this means frequently yielded variants of Type II. The directional change from Type I to II was observed in all species studied. No change took place in cultures less than 3 weeks old.

Type II variants obtained during single-spore analysis in no case (278 tested) reverted to Type I. Only when employing mass transfer tech-

nique was a Type II culture obtained that subsequently reverted to Type I. This was interpreted as a segregating out of Type I nuclei that had been perpetuated from the outset by mass transfer.

Many variants, when compared from a taxonomic standpoint with their parent cultures, could no longer be considered as the same species nor occasionally even to be in the same section as their parents. A variant of *F. graminearum* had to be reclassified as *F. sublunatum* var. *elongatum*, Three variants obtained from *F. culmorum* were *F. reticulatum*, f.1, *F. sambucinum*, and *F. flocciferum*. A variant of *F. equiseti* could not be classified with any existing species. A crossing of sectional lines from Gibbosum to Roseum was necessitated by a variant of *F. scirpi*.

The pathogenicity of each isolate and variant was determined on wheat seedlings using greenhouse pot tests. All isolates of all species except *F. scirpi* and *F. sambucinum* were pathogenic to some extent. A culture of *F. sambucinum* that arose as a variant from *F. culmorum* was slightly virulent. The variants of cultural Type II were significantly less parasitic than their parental types in all cases. Within a single species such as *F. graminearum* or *F. culmorum*, there was exhibited a range of pathogenicity similar to that of the whole group of species and sections.

It is felt that the evidence presented demonstrates the interrelationship of the Fusarium isolates of the sections Gibbosum, Roseum, and Discolor, both on a taxonomic and a pathogenic basis; and further it tends to substantiate the grouping of all these into one species as advocated by Snyder and Hansen.

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TRANSMISSION OF THE MILD STREAK VIRUS OF BLACK RASPBERRY[1]

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Since mild streak of black raspberry was first described by Dodge and Wilcox (8), many workers have considered this disease to be caused by a virus (1, 4, 7, 8, 12, 13, 14, 15, 16, 17, 18), although experimental evidence of transmission was not presented. Bennett (1) attempted to transmit mild streak with several species of insects but his tests were all negative. In view of the economic importance of mild streak in many black raspberry growing districts, including certain plant producing areas, it was highly desirable to secure definite proof of the experimental transmission of the disease under controlled conditions. The present paper reports experimental transmission of mild streak in black raspberry by cane grafting and by means of dodder.

MATERIALS AND METHODS

Healthy black raspberry (*Rubus occidentalis* L.) layers were obtained from three sources: (1) the commercial variety Logan from an isolated black raspberry patch relatively free from mild streak near Keedysville, Maryland; (2) a hybrid, Cumberland × Evans (referred to hereafter as hybrid plants), from an isolated patch at the University of Maryland farm at College Park, Maryland; (3) the commercial variety Dundee from an isolated patch in a home garden near College Park, Maryland. Diseased Cumberland black raspberry plants were obtained from naturally infected plantings in Western Maryland. In certain instances detached canes of both healthy and diseased field-grown plants from the same locality were used in graft transmission tests.

Grafts between healthy and diseased plants were made by excising tissues of the canes down to the xylem and binding them securely together with Florotape. The usual union extended about 5 to 8 cm. longitudinally along the cane. The test plants were defoliated to increase movement of virus from the source plants; as Hildebrand (9) has shown, creation of a carbohydrate deficit promotes translocation of virus toward deficient areas.

"Dodder grafts" were made by a simplification of Cochran's technique (2, 3) utilizing *Cuscuta subinclusa* D. & H. The test plants in "dodder grafting" experiments were defoliated in the same way, and for the same reason, as in the approach grafts.

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Aphids (*Amphorophora sensoriata* Mason) were collected from field-grown healthy and diseased Logan plants in Western Maryland. Sweepings for other insects were also made in a heavily infected Logan patch in the same area. The insects so obtained were identified by Dr. P. W. Oman[3] as leaf hoppers (*Deltocephalus flavicosta* Stal.) and spittle bugs (*Philaenus leucophthalmus* L.).

Throughout all experiments the cane streaking symptom was used as an indication of transmission. Leaf-hooking was not a reliable indication of mild streak in the greenhouse, and the vein-clearing symptom (Fig. 1, A) appears inconsistently on infected plants. The fruit symptom could not be used because berries are produced only during a short period in the greenhouse (Fig. 2, C).

RESULTS

Graft transmission. In a preliminary experiment (June 22, 1946), 10 healthy Logan plants were approach-grafted to 10 diseased Cumberland plants in the greenhouse. Controls included 10 approach grafts between apparently healthy plants of either Logan or Dundee. Both of these varieties are known to be susceptible to mild streak (16). About two months after grafting, cane streaking began to appear on five of the test plants, and four of the Logan control. At the end of the summer the grafts were broken, and all plants were heavily pruned and then placed out of doors from September 21, 1946, to January 20, 1947. Two months after the plants had been returned to the greenhouse, three more of the test plants showed streaking on the new vigorously growing shoots. All plants were observed until May 28, 1947, but cane streaking did not appear on any of the remaining plants. Several other plants from this supposedly healthy stock, but which were not used in the experiment, also developed mild streak at this time. It is evident, therefore, that some residual infection was present at the beginning of the experiment.

Thirteen healthy hybrid plants, three healthy Dundee plants, and four healthy Logan plants, all of which were defoliated except for a few terminal leaves, were approach-grafted to 20 diseased Cumberland plants. Controls consisted of the same number of plants of each of the same varieties treated similarly, and approach-grafted to 20 healthy hybrid plants. The results are given in table 1. All plants were held until November 25, 1947. None of the remaining plants developed cane symptoms. Of those that became infected, streaking appeared first on some plants on the new lateral growth below the union, on other plants first at the base of canes other than the grafted ones, and on still others on both grafted and non-grafted canes of the same plant.

In another experiment, transmission was obtained by approach-grafting 10 healthy Dundee plants, which were defoliated except for a few terminal leaves, to 10 diseased plants. Controls consisted of 10 approach grafts

[3] United States Department of Agriculture, Bureau of Entomology and Plant Quarantine, Division of Insect Identification, Washington, D. C.

between healthy Dundee plants treated similarly. All grafts were made on October 10, 1947. No symptoms were observed on any of the test plants by December 15, 1947, at which time all grafts were broken, each plant was pruned heavily, and then all plants were subjected to dormant conditions (winter temperatures) until February 2, 1948. New vigorous growth was resumed and cane streaking was observed on one test plant 68 days later, and by May 11, 1948, a total of 9 of the 10 test plants showed cane symptoms. Streaking was not observed on any of the control plants.

Some field-grown plants were observed in which the canes were heavily streaked, while in others streaking was moderate, and in still others so

TABLE 1.—*Transmission of mild streak in black raspberry by grafting*

Graft number[a]	Date graft was made	Number of days before streaking appeared on test plants[b]
1	March 26, 1947	39
2	do	77
3	do	77
4	do	39
5	do	77
6	do	77
7	do	103
8	do	77
9	do	77
10	do	39
11	July 5, 1947	90
12	do	75
13	do	no symptoms
14	do	do
15	do	do
16	do	do
17	do	75
18	do	75
19	do	90
20	do	75

[a] Plants 1–13 were healthy hybrids, 14–16 were healthy Dundee, and 17–20 were healthy Logan. All were grafted to diseased Cumberland plants.

[b] Controls, consisting of 20 healthy plants grafted to 20 healthy plants, remained negative.

faint as to be barely discernible. Five cuttings of the current-season canes from plants of each of these symptom types were approach-grafted to healthy Logan plants in the greenhouse. Five cuttings from healthy Logan plants in the field were approach-grafted to five healthy Logan plants in the greenhouse as controls. All grafts were made on August 2, 1946. On December 3, 1946, the plants were pruned heavily and placed out-of-doors where they remained for the duration of the experiment. On June 21, 1947, the plants were rated as to the severity of streaking (Table 2).

Dodder transmission. Thirty diseased plants were "dodder-grafted" (3) to 30 healthy plants in the greenhouse with *Cuscuta subinclusa.* The test plants were defoliated except for a few terminal leaves, and only the stem area required for grafting was defoliated on source plants. Thirty

TABLE 2.—*Graft transmission of mild streak, from field cuttings of Logan black raspberry differing in severity of symptoms, to healthy plants of the same variety*

Severity of streak in field plants from which affected canes were taken	Number of plants infected[a] and severity of symptoms			
	Mild	Medium	Pronounced	Apparently healthy
Mild	1	2	1	1
Medium	1	1	1	2
Pronounced	1	0	2	2
Controls	0	0	0	5

[a] Five plants were grafted in each group.

healthy plants "dodder-grafted" to 30 healthy plants in a similar manner were used as controls. The results are shown in table 3. The first indica-

TABLE 3.—*Transmission of mild virus in blackberry by "dodder grafting"*

Graft number	Varieties grafted[a]		Number of days before appearance of cane symptoms[b]
	Test plant	Source plant	
1	Cumberland × Evans	Logan	67
2	do	do	no symptoms
3	do	do	67
4	do	do	no symptoms
5	do	do	84
6	do	do	no symptoms
7	do	do	do
8	do	do	67
9	do	do	no symptoms
10	do	do	do
11	Logan	do	do
12	do	do	72
13	do	do	no symptoms
14	do	do	do
15	Dundee	do	do
16	do	do	do
17	do	do	do
18	Cumberland × Evans	Cumberland	do
19	do	do	do
20	do	do	do
21	do	do	do
22	do	do	do
23	do	do	do
24	do	do	do
25	do	do	do
26	do	do	do
27	do	do	54
28	do	do	54
29	do	do	54
30	do	do	no symptoms

[a] Grafts 1–10 were made on April 11, 1947; 11–20 on July 7, 1947, and 21–30 on August 28, 1947. All plants were observed for five months before discarding.

[b] Dodder grafts between 30 pairs of healthy plants of the same variety as the test plants were used as controls which remained negative throughout the experiment.

tion of transmission was a hooking of the younger leaves (Fig. 1, C). Several weeks later slight cane lesions developed on the new basal growth.

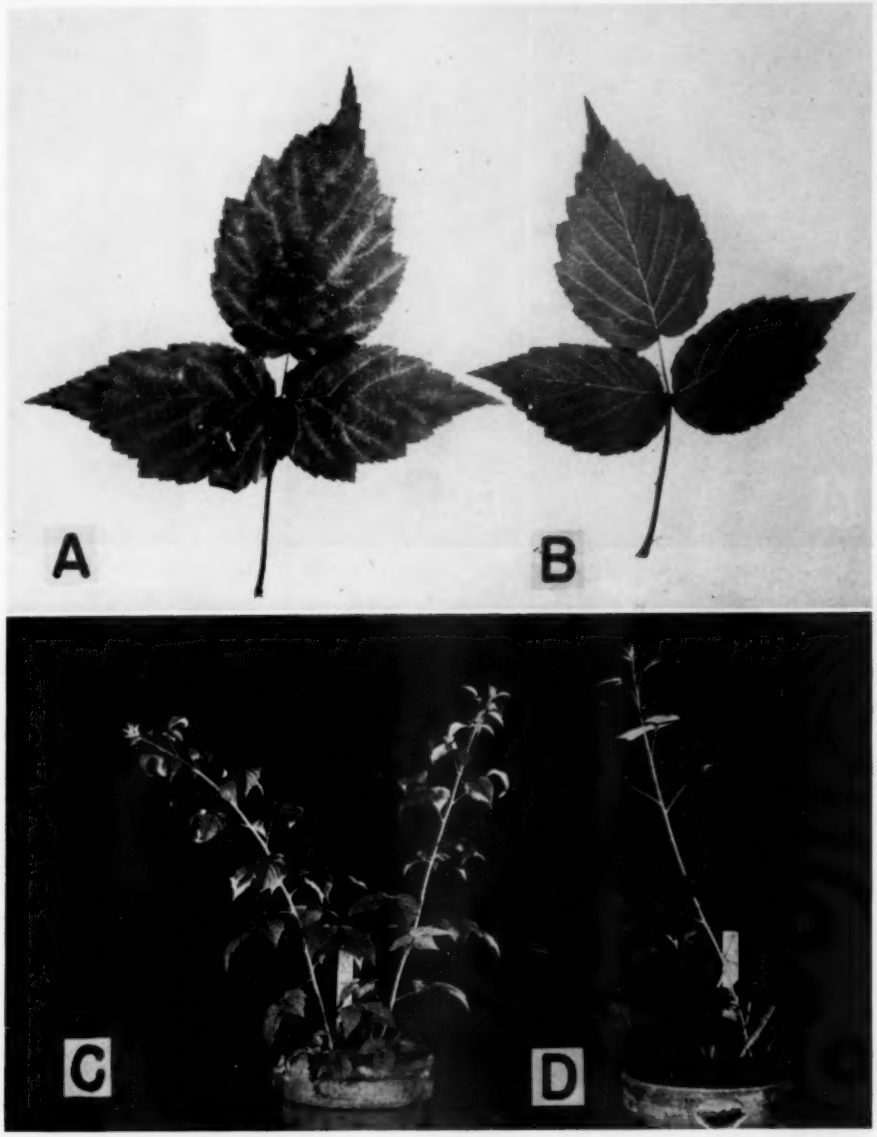

FIG. 1. A. A leaf taken from a diseased hybrid (Cumberland × Evans) plant showing the vein clearing symptom. B. A leaf taken from a healthy hybrid plant. C. Hooked leaves on a diseased plant of the Cumberland variety. D. Normal, flat leaves on a healthy hybrid plant.

Further development consisted of an increase in the amount of these cane streakings (Fig. 2, A).

Since the canes involved in "dodder-grafting" had to be bound tightly,

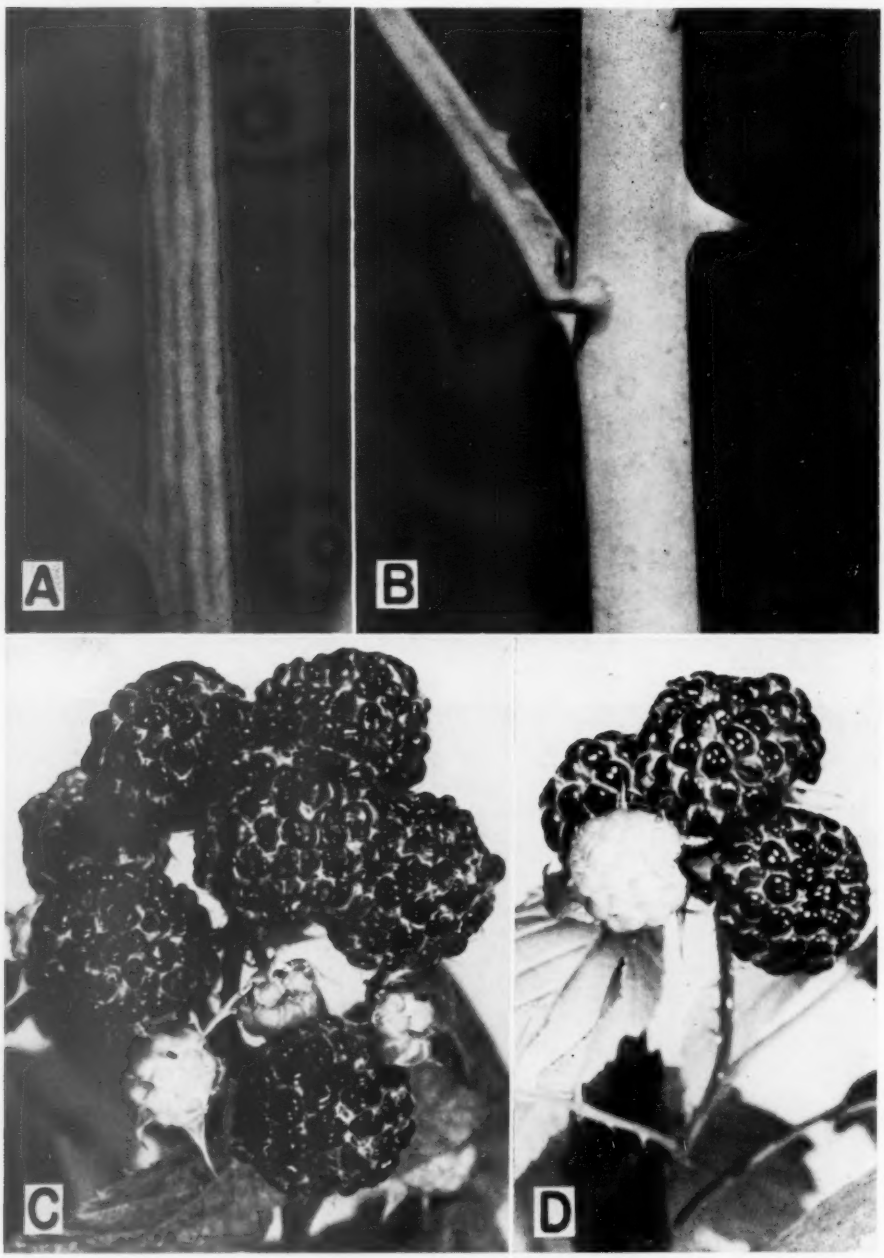

FIG. 2. A. A portion of a cane (Cumberland variety) severely infected with mild streak. B. A portion of a healthy cane (Cumberland × Evans), showing the clean, white appearance of the bloom. C. Berries from a diseased Cumberland plant, showing the over-all dull appearance of the fruit and the sunken drupelets, approximately 1×. D. Berries from a healthy Logan plant, showing the fullness of each drupelet and the healthy shiny appearance of the fruit in general, approximately 3/4×.

some injury may have occurred at the points of binding so that an unintentional union could have been made between the test and source plants. In order to test this possibility of unintentional grafting, 10 healthy plants were attached to 10 diseased plants in the manner described for "dodder-grafting" but void of dodder. There was no evidence of union between any of the canes at the end of three months, and no symptoms occurred on any of the test plants within eight months.

Mechanical transmission. Three healthy Logan, three healthy Dundee, and four healthy hybrid plants were inoculated mechanically with freshly prepared sap from leaves of diseased plants. The expressed sap was obtained at greenhouse temperature (approximately 85° F. by crushing freshly harvested leaves from diseased plants in a sterilized mortar. A 6-inch pot label, wrapped at the blunt end with sterile gauze, was used to inoculate the plants. Carborundum[6] was mixed with the inoculum, and 10 leaves of each test plant were inoculated at random. Ten healthy plants of the same varieties were similarly inoculated as controls, with expressed sap prepared in the same manner from leaves of healthy plants. Inoculations were made on June 20, 1947, and final observations were made on November 7, 1947. No symptoms were apparent at any time on either the test or control plants.

Insect transmission. Stem aphids (*Amphorophora sensoriata*) were collected on June 20, 1946, from a planting of Cumberland black raspberries which contained both healthy and diseased plants. In the greenhouse, seven hours later, aphids which had been feeding on apparently healthy plants in the field were caged on healthy Dundee and Logan plants and diseased Cumberland plants. Aphids which had been feeding on diseased plants in the field were caged on healthy Dundee and Logan plants. All insects began to feed within an hour after transfer and were permitted to feed for five days. Sub-colonies were made until a total of 44 healthy Dundee and Logan plants had been exposed to insects which had been feeding on diseased plants, and 21 healthy Dundee and Logan plants had been exposed to insects which had been feeding on healthy plants. Attached tip layers were rooted from all the test plants at the time the insects were caged on them. Both layers and parent plants were held through the following spring of 1947, but no symptoms were observed on any of the plants.

Insect sweepings were made in a Logan black raspberry patch at Keedysville, Maryland, on June 27, 1947. This planting was known to be 90 per cent infected with mild streak disease. Within five hours the insects were transported to the greenhouse where they were treated with sufficient chloroform vapor to render them inactive for 10 minutes in order to segregate them into generic groups for counting. The insects used were leafhoppers (*Deltocephalus flavicosta* Stal.) and spittle bugs (*Philaenus leucophthalmus*

[6] Grade No. 600, Manufactured by the Carborundum Company, Niagara Falls, New York.

L.). Twenty Logan and Dundee plants growing in the greenhouse under one large cage (mosquito netting) were exposed to 62 of the leafhoppers and 95 of the spittle bugs. Controls consisted of 20 Dundee and Logan plants under a similar cage from which insects were excluded. No symptoms appeared on any of the plants by November 15, 1947.

DISCUSSION

The mild streak disease of black raspberry has been described repeatedly and, although evidence of experimental transmission has been lacking, the disease has usually been assumed to be of virus nature. A preliminary report (10), however, presented evidence of graft transmission. In the present study the disease was graft-transmitted from infected black raspberry plants to healthy black raspberry plants in 35 of 45 attempts. The results of an early experiment in which some of the controls were infected are not included in the figures mentioned, since it seems likely that the supposedly healthy controls used in this experiment were already infected by natural means before the experiment was begun. Transmission was obtained by "dodder grafting" with *Cuscuta subinclusa* in 8 of 30 attempts. In view of the type of symptoms occurring in diseased plants, together with the demonstration of graft transmissibility, it is now considered justifiable to regard mild streak as definitely of virus nature.

The wide variation in severity of symptoms in the field, and in plants artificially inoculated, has suggested the possibility of strains of the mild streak virus. The data obtained, however, showed that the degree of severity of cane streaking was not maintained when the virus was introduced into healthy plants by grafting. A more extensive study along this line should be undertaken.

On the basis of field observations, Rankin (13), Slate et al. (15), Cooley (4, 5, 6), and Jeffers and Woods (11) suggested that the mild streak virus may be transmitted by an insect. Bennett (1) found that *Amphorophora sensoriata* does not transmit the disease in Michigan, and Cooley (6) suggested that the vector is not an aphid. In the present study an aphid (*A. sensoriata*), a leafhopper (*Deltocephalus flavicosta*), and the spittle bug (*Philaenus leucophthalmus*) were tested as possible vectors of mild streak, but in none of the tests did these insects transmit the disease. An extensive program involving field collections and testing of insects at short intervals throughout the spring and summer should be conducted with the above mentioned insects before finally excluding them from a list of possible vectors.

The expressed sap from the leaves of black raspberry plants is readily oxidized at temperatures of 70° F. and higher (1). This might explain failure to obtain mechanical transmission in the present investigation, although other factors may also be involved.

SUMMARY

The mild streak disease was found to be graft-transmissible from dis-

eased Cumberland and Logan black raspberry plants to healthy Logan, Dundee, and hybrid plants (Cumberland × Evans) in 43 of 55 attempts.

Transmission was obtained in 8 of 30 attempts by "dodder grafting" with *Cuscuta subinclusa* from diseased to healthy black raspberry plants.

The virus nature of the mild streak disease of black raspberry is now considered to be established.

Existence of strains of the virus was not demonstrated since variations in the degree of severity of cane symptoms occurring on the variety Logan in the field were not transmitted consistently by grafting to healthy Logan plants in the greenhouse. *Amphorophora sensoriata* Mason, *Deltocephalus flavicosta* Stal., and *Philaenus leucophthalmus* L. did not transmit the virus to black raspberry plants under the experimental conditions employed.

No transmission was obtained by mechanical inoculation of healthy black raspberry plants with freshly expressed sap from the leaves of mild streak diseased black raspberry plants.

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THE INFLUENCE OF NITROGEN SOURCE ON THE DEVELOPMENT OF STEM RUST OF WHEAT[1]

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INTRODUCTION

It is well established that environmental conditions may have a pronounced effect on the stem rust reaction of wheat seedlings (1, 11). With certain combinations of host and physiologic race of rust, increasing temperatures and light intensities will shift the infection type from a resistant to a susceptible class. The present work was undertaken to determine whether shifts in infection type could be induced through changes in nutrient balance and whether such shifts would explain some of the interactions between host and parasite.

Throughout this study, the wheat varieties Mindum (C. I. 5296), Marquis (C. I. 3641), and Thatcher (C. I. 10003) were inoculated with physiologic race 56 of *Puccinia graminis tritici* because these varieties are resistant, susceptible, and mesothetic, respectively, to this race. The method of inoculation and criteria of rust infection were those of Stakman, Levine, and Loegering (19). Resistant reactions are indicated by infection types 0, 1, and 2, the susceptible class includes infection types 3 and 4, while the mesothetic reaction is designated X. The individual infection types are further designated by plus or minus signs indicating that the infection type approaches the maximum or minimum for the type. This is based on the amount of sporulation, necrosis, or chlorosis.

NUTRITIONAL STUDIES IN SAND CULTURE

Plants of the three varieties were grown in washed quartz sand contained in glazed crocks of 2000-cc. capacity. A 500-cc. Erlenmeyer flask, serving as a reservoir, was attached by rubber tubing to an outlet at the base of each crock. By alternate inversion of the flask above and below the level of the crock, nutrient solutions could be rapidly drained from the sand at intervals during the day, thus promoting a favorable oxygen tension for root development. This arrangement also permitted a ready change of nutrient every five days.

Each crock accommodated ten plants of each variety, and three replicates were used for each treatment. Nutrients were applied after the primary leaves had broken through the coleoptile. The major elements were supplied as three-salt solutions of equal osmotic pressure, as outlined by Livingston (12); the nature of the three salts composing a particular nutrient solution

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[2] The writer is indebted to Miss Helen Hart for suggestions and criticisms during the course of the work and preparation of the manuscript.

was governed by the balance of ions desired for the treatment. Nitrogen in all cases was supplied as nitrate. Minor elements were furnished as their sulfates; iron, 2 ppm; boron, 0.5 ppm; copper, manganese, and zinc, 0.08 ppm. The wide diversity in composition of the 38 nutrient solutions tested

TABLE 1.—*Composition of 38 three-salt solutions and the rust infection types on Mindum, Marquis, and Thatcher wheats inoculated with race 56 of Puccinia graminis tritici*

Solution	K	P	Ca	N	Mg	S	Rust infection types on		
	P.p.m.						Mindum	Marquis	Thatcher
1	95	75	200	140	305	395	1 – to 1 +	3 – to 3	1 = to 1 +
2	85	65	435	305	105	140	0; to 1 ++	3 – to 3 +	1 – to 1 +[a]
3	200	160	110	80	325	425	0; to 1	3 – to 3	1 – to 1[a]
4	180	140	285	200	175	230	0; to 1	3 – to 3	1 – to 1 ++
5	155	120	440	310	50	70	0; to 1	3 – to 3 +	1 to 3 = to 3 +
6	260	205	275	195	110	145	1 – to 1 +	3 – to 3 ++	1 to 3 = to 3 +
7	470	370	120	85	120	160	1 – to 1 +	3 – to 3 +	1 = to 1 +
8	450	355	190	135	60	75	0; to 1	3 = to 3 +	1 = to 1 +
9	75	600	155	110	235	60	0; to 1 ++	3 = to 4 +	3 = to 3 +
10	75	230	375	260	90	60	0; to 1	3 – to 3 +	3 – to 3 +
11	150	590	90	55	230	120	0; to 1 ++	3 to 4 ++	3 = to 4 –
12	140	340	215	150	130	115	0; to 1 +	3 – to 4	3 = to 3 ++
13	140	110	365	250	45	115	0; to 1 ++	3 to 4 +	3 – to 3 ++
14	220	250	230	160	90	180	0; to 1 +	3 to 4 –	3 = to 3 +
15	365	230	90	50	90	300	0; to 1 ++	3 – to 4 +	3 = to 3 +
16	370	120	150	105	45	300	0; to 1 ++	3 to 4 –	3 = to 3 +
17	100	330	210	35	320	425	1 – to 1 ++	3 to 4 +	3 = to 3 +
18	230	170	110	80	325	435	0; to 1	3 – to 4 +	3 – to 3 +
19	175	420	270	65	160	215	1 to 1 ++	3 – to 4	3 = to 3
20	260	410	270	95	110	145	1 – to 1 ++	3 to 4 –	3 = to 3 +
21	490	150	95	175	115	155	0; to 1 +	3 – to 4 +	3 = to 3
22	430	280	180	160	55	75	0; to 1 +	3 to 4 +	3 = to 3
23	140	200	125	265	210	55	0; to 1	3 – to 3	3 = to 3 –
24	135	575	360	100	85	60	0; to 1 +	3 – to 3 +	3 = to 3 –
25	275	110	70	260	210	110	0; to 1 +	3 = to 3	3 – to 3
26	275	350	220	155	130	110	0; to 1	3 = to 3	3 = to 3 –
27	295	600	380	55	45	120	0; to 1	3 – to 4 –	3 – to 3 +
28	430	350	220	105	85	180	0; to 1 +	3 – to 4 –	3 = to 3
29	750	120	75	100	90	300	0; to 1	3 – to 3	3 – to 3 +
30	760	240	155	54	45	310	0; to 1	3 = to 3 +	3 = to 3 +
31	80	650	160	20	250	130	0; to 1	3 = to 3 ++	3 = to 3 –
32	90	250	410	30	100	330	0; to 1 =	3 = to 3 –	3 = to 3 –
33	150	650	65	55	250	65	0; to 1 +	3 = to 4 –	3 – to 3
34	165	415	260	60	160	210	0; to 1 +	3 = to 3 +	3 – to 3 +
35	185	155	475	65	60	380	0; to 1	3 – to 3 +	3 – to 3 –
36	260	295	270	100	115	230	0; to 1	3 = to 3 –	3 – to 3
37	395	270	90	155	110	75	0; to 1 +	3 – to 3 +	3 – to 3
38	400	145	175	155	55	145	0; to 1	3 – to 3 +	3 = to 3 –

[a] Rust reactions based on only two replicates.

is shown in table 1, in which concentration of the six major elements is given, for convenience, in parts per million.

The plants were grown at temperatures above 80° F., and inoculated with rust 20 to 25 days after planting. Notes on rust development were obtained 13 or 14 days after inoculation. Because of limitation in space, only eight solutions were tested in a single experiment.

Plant vigor was relatively uniform and normal. Solutions 9, 14, 36, 37,

and 38 produced slightly chlorotic plants. The concentrations of calcium and phosphorus in solutions 24 and 27 caused a slight tip-burn.

Despite the variation in composition of nutrient solutions there was only slight evidence that rust development was affected (Table 1). Mindum was resistant and Marquis was susceptible in all cases. Thatcher was moderately susceptible (infection type 3) except during a period of low light intensity in late winter (solutions 1 through 8). In solutions 5 and 6, susceptible reactions developed on some of the plants, although plants in the remainder of the six treatments tested at the time were resistant. Solutions 5 and 6 contained relatively high proportions of nitrogen, but nitrogen was not the only factor involved since Thatcher grown in solution 2, higher nitrogen but lower potassium and phosphorus, was resistant.

These results suggested that light and possibly temperature might tend to mask the effects of nutrition. Since only nitrate had been used as a nitrogen source in sand culture and because of the differential assimilation of ammonium and nitrate nitrogen at various pH levels (21), combinations of these two factors, in conjunction with temperature, were tested.

THE INFLUENCE OF NITROGEN AND TEMPERATURE ON RUST DEVELOPMENT

Five plants each of Thatcher and Mindum wheat were grown in a mixture of 3 parts sand and 2 parts greenhouse soil in a single 4-in. pot. After the seedlings had emerged, 100 cc. of solution containing 400 ppm. of nitrogen as one of the following salts was applied every other day: potassium nitrate, KNO_3; calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$; ammonium nitrate, NH_4NO_3; ammonium sulfate, $(NH_4)_2SO_4$. The check consisted of similar applications of a solution of 0.5 gm. of potassium phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), and calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$). Three replicates of each treatment were maintained at pH 8.0–9.5 and three at pH 4.0–4.5. The plants were placed at different temperatures on the first day nitrogen was supplied. Six days later they were inoculated with race 56 of *Puccinia graminis tritici*, and final rust observations were made 15 to 19 days after inoculation.

Rust reaction at low temperature. Plants supplied nitrogen differed only slightly in vigor. There was a slight tendency for nitrate-treated plants to tiller more than ammonium-treated plants in the first experiment, but in later experiments no differences were noted. The check plants receiving low nitrogen were distinctly stunted and chlorotic. The rust reaction of Mindum was not altered by nitrogen source; all plants were resistant (0; to 1 infection types), although the check plants had fewer pustules.

The check plants of Thatcher wheat, and those supplied ammonium sulfate, were resistant to rust (0; to 1 infection types). Plants supplied potassium nitrate were mesothetic (X to X++ infection types) approaching very closely to complete susceptibility (Table 2 and Fig. 1). Calcium nitrate

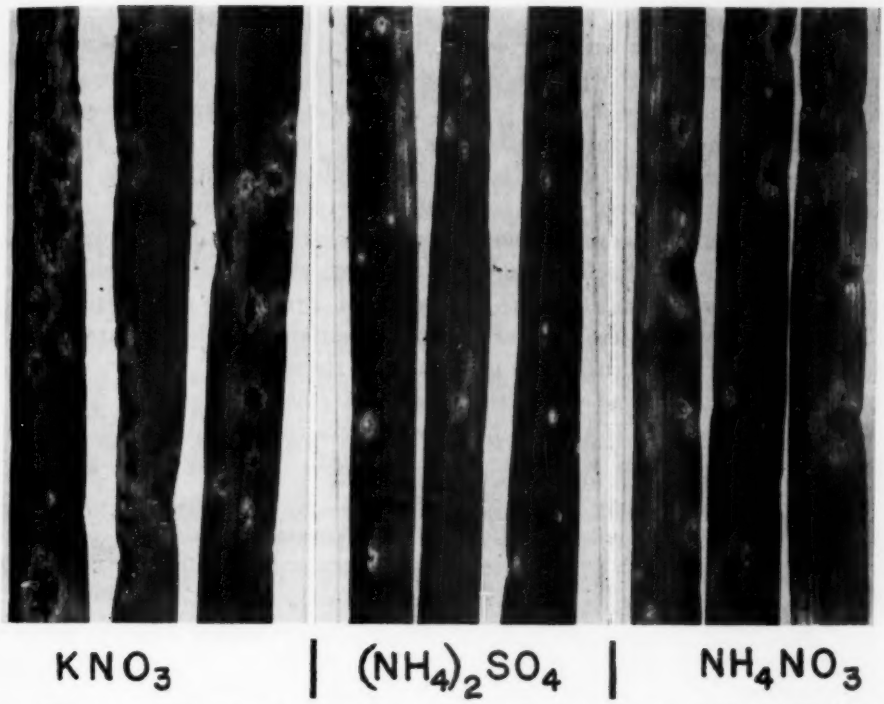

FIG. 1. Infection types produced by race 56 of *Puccinia graminis tritici* on Thatcher wheat supplied three different nitrogenous salts. KNO_3; x to x++ infection type. $(NH_4)_2SO_4$; 0; to 1 infection types. The center leaf has two pustules of powdery mildew. NH_4NO_3; x = to x infection types.

treated plants were slightly more resistant (X– to X++ infection types). The application of 400 ppm. of nitrogen as ammonium nitrate (200 ppm. of NH_4^+ and 200 ppm. NO_3^-) resulted in plants intermediate in reaction between ammonium and nitrate plants. Some leaves were completely resistant and some were mesothetic, but with evident resistance. Since pH did not alter rust reaction, the results of all pH levels for two experiments are grouped in table 2.

In later tests, comparisons were made of the nitrogenous constituents of

TABLE 2.—*The influence of nitrogen source on the rust reaction of Mindum and Thatcher wheats inoculated with race 56 of Puccinia graminis tritici and grown at 65°–75° F.*

Treatment	Rust infection type on:	
	Mindum	Thatcher
KNO_3	0; to 1++	x+ to x++
$Ca(NO_3)_2 \cdot 4H_2O$	0; to 1++	x= to x++
NH_4NO_3	1 to 1++	0; to 1++ (22 leaves) x= to x+ (30 leaves)
$(NH_4)_2SO_4$	0; to 1++	0; to 1+
Check	0; to 1+	0; to 1+

Thatcher and Mindum wheats under these conditions. Ten pots of each variety were supplied with nitrogen salts as outlined above, but no attempt was made to adjust the pH of the sand-soil mixture. Eleven days after first application of the salts, 2 pots of each treatment were removed from the greenhouse bench and inoculated with race 56 of stem rust. Ten days after inoculation the leaves of the inoculated plants began to fleck. In the evening, 30 gm. of leaves of the same age were removed from the remaining 8 noninoculated plants and immediately autoclaved at 5-lb. pressure for 5 min. The leaves were dried at 60° C. and weights were recorded. The leaves were harvested at the time of first visible symptoms of rust infection since it was believed, from other evidence, that the metabolic state of the leaves at this time establishes the ultimate rust reaction. Final rust reaction of inoculated plants was estimated 10 days after flecking.

Total nitrogen was determined by the micro-Kjeldahl method after preliminary reduction with zinc. Soluble reducing substances were de-

TABLE 3.—*Rust reaction and composition of Thatcher and Mindum wheats grown at low temperatures and inoculated with race 56 of Puccinia graminis tritici*

Treatment[a]	Thatcher			Mindum		
	KNO_3	$(NH_4)_2SO_4$	NH_4NO_3	KNO_3	$(NH_4)_2SO_4$	NH_4NO_3
Rust reaction	x – x++	0; – 1	0; – 1 –[c], x – x++	0; – 1	0; – 1	0; – 1
Dry weight (per cent)	14.2	13.8	14.3	17.0	15.6	15.7
Content[b] of						
Total N	45.8	51.1	51.9	43.5	48.6	50.7
Protein N	37.7	41.6	42.3	36.6	39.9	41.5
Ammonia N	0.14	0.57	0.40	0.10	0.36	0.28
Amide N	0.46	0.79	0.60	0.18	0.26	
Amino N	2.98	4.16	3.64	2.69	3.22	2.95
Total carbohydrate	76.0	66.6	60.4	93.2	85.6	78.4
Reducing substances	8.2	14.8	13.6	9.0	17.0	16.0

[a] 400 ppm. nitrogen.
[b] Expressed as mgm. per gm. dry weight.
[c] Two leaves with resistant reaction.

termined by a modification of the Shaeffer-Sogomyi (10) procedure and total soluble carbohydrate by the same method after acid hydrolysis. Protein nitrogen was estimated in the micro-Kjeldahl apparatus on the residue from alcohol extraction of sugars. Amide and ammonia nitrogen were extracted with water and determined by the method of Pucher *et al.* (16). Amino nitrogen in the same extract was determined by the van Slyke procedure.

The results of analysis, and the rust reactions of inoculated plants, are presented in table 3. Thatcher wheat grown with ammonium nitrate did not have so high a degree of resistance as in previous experiments, as only two leaves were resistant. Infection of the remainder of the leaves was of the mesothetic class, but the number of resistant pustules was greater than in plants supplied with potassium nitrate. The analytical results indicate

that none of the leaf fractions examined can be definitely linked with the factors responsible for resistance and susceptibility in this instance. There is no correlation between rust resistance and total or protein nitrogen. Resistant leaves of Thatcher have a higher content of both forms of nitrogen than do the leaves of susceptible plants, but plants supplied ammonium nitrate have still greater amounts of total and proten nitrogen even though the leaves are slightly more resistant. A similar but inverse relationship is found with total soluble carbohydrates.

Soluble nitrogen levels induced by the various treatments form a pattern that follows the degree of resistance of the leaves. Resistant leaves have the highest content of ammonia, amide, and amino nitrogen, while moderately susceptible leaves have approximately 1/4 the ammonia, 1/2 the amide nitrogen, and 2/3 the amount of amino nitrogen. Plants supplied with ammonium nitrate are intermediate with respect to these compounds. However, the same relative differences in soluble nitrogen can be observed in Mindum, but without a corresponding change in rust reaction.

The trends for soluble reducing substances are the same as for soluble nitrogen components, but this may indicate a nonspecificity of the sugar reagents which becomes apparent only when the ratio of amino acids to monosaccharides is low. The lower content of nitrogenous compounds and higher content of sugars in Mindum can be ascribed to a varietal difference in nitrogen metabolism.

Although it is impossible to single out one fraction as responsible for protoplasmic resistance of wheat to stem rust, the possibility remains that the balance of metabolites in the leaves of Thatcher determines the resistance of this wheat to race 56, but that in Mindum wheat another leaf constituent, not revealed by these data, exercises more influence in controlling rust reaction to this race.

Rust reaction at high temperatures. Attempts to grow plants under the same conditions at temperatures between 80° and 90° F. were not successful. The plants grew abnormally fast and lacked the vigor of the plants grown at low temperature. Although rust readings were made, the condition of the plants does not justify serious consideration of the results. Thatcher in all cases was susceptible (infection types 3 to 4), while Mindum was resistant (infection types 0; to 1).

FIELD EXPERIMENTS

Plots of three rows each of Mindum, Marquis, and Thatcher wheat were planted on a light sandy loam. Each plot was eight feet square with 2-ft. alleys between plots. Border rows were inoculated with race 56, to supply inoculum for the natural spread of rust to the plots. Treatments consisted of 200 lb. per acre of calcium nitrate, and 270 lb. per acre of urea, ammonium sulfate, or ammonium nitrate applied at time of planting. Each treatment was replicated three times.

Plots treated with nitrogen were uniformly more vigorous than the

checks. The plants were taller, tillered well, and were darker green than control plants. There was no influence of nitrogen on the percentage or type of rust infection. Marquis was susceptible, averaging 30 per cent infection, while Mindum was resistant.

Thatcher wheat, normally mesothetic in the seedling stage, developed only a trace of stem rust since this variety has a high degree of adult plant resistance. This is perhaps largely due to morphogical characteristics and would tend to mask any change in the protoplasmic resistance.

DISCUSSION

Most investigations concerning nutrition of wheat in relation to the development of stem rust have been made in the field and, as Gassner (2) and Stakman and Aamodt (18) have pointed out, the effects of fertilizers are largely indirect, since photoplasmic resistance, the cellular incompatibility between host and parasite, has not been altered. In general, the addition of nitrogenous compounds delays maturity, increases density of stand, and promotes succulence, conditions conducive to increasing the number of infections but not necessarily to change in infection type.

Nutritional studies on seedlings in the greenhouse have yielded negative results (25), except in instances where experimental conditions have visibly altered the vigor of the host. It is well known that vigor of the host is directly correlated with the development of rust and if the general well-being of the host has been affected, it can not be shown with certainty that the normal protoplasmic factors governing rust resistance have been changed. Instead, secondary factors may be operative. Darley and Hart (1), however, reported that low nutrient levels produced relatively low infection types on Kenya wheat, while high nutrient levels raised the infection types.

The results presented here indicate that failure to find a positive influence of nutrition on rust development may be associated with interactions of temperature, nitrogen source, and the host-parasite combination. These interactions have been investigated in connection with Fusarial diseases by Walker and recently summarized (23). The resistance of Thatcher wheat to race 56 of stem rust is analogous with the "type B" resistance possessed by some cabbage varieties to *Fusarium oxysporum* f. *conglutinans*. At high temperatures nitrate nitrogen did not influence rust reaction even though the concentrations ranged from 20 to 305 ppm. At low temperatures, plants supplied 400 ppm. of nitrate nitrogen were moderately susceptible but 200 ppm. of nitrate nitrogen lowered the susceptibility, even though the plants received an additional 200 ppm. of ammonium nitrogen. Plants receiving 400 ppm. nitrogen in the form of the ammonium ion were completely resistant. Mindum wheat corresponds to cabbage varieties with "type A" resistance in that none of the environmental factors investigated caused shifts in rust reaction. Thatcher and race 56 of stem rust appear to be in delicate balance at temperatures between 60° and 75° F., and only at that temperature can the balance be upset by nutrition.

Gassner and his coworkers (3, 4, 5, 6) reported that increasing amounts of nitrogen raised the susceptibility of wheat to stripe and leaf rusts. The varieties most subject to variation were the moderately susceptible or moderately resistant types. Later these workers concluded that susceptibility was correlated with the concentration of protein, but not with soluble nitrogen. Mains, on the other hand, working with several rusts (13), thought that obligate parasitism and resistance to rust were dependent on the availability of carbohydrates. The present analysis of the wheat varieties grown at low temperatures indicates that changes of rust reaction as a result of nitrogen nutrition are accompanied by changes in both carbohydrate and nitrogenous compounds, particularly the individual soluble nitrogen compounds.

These results are in accord with the literature on nitrogen metabolism (15) and it appears impossible to predict, from experiments in which disease resistance has been altered by manipulation of one or two environal conditions, which class of leaf constituents is fundamental to the change in disease expression. For example, protein content of the cell has been shown to be reflected in the respiration rate (8, 9, 17, 26), photosynthetic activity (9), ascorbic acid content (27), organic acids (22, 26), and salt uptake (20). Protein content, in turn, is regulated by available carbohydrate (17, 26), supply of potassium and phosphorus (8, 17), and the presence of "sinks" in other plant parts (24). It is therefore necessary to investigate a wide number of leaf constituents and to consider the influence of interlocking metabolic systems before implicating one process or group of compounds (protein, sugar, ammonia (7), phenols (14)) as the source of rust resistance.

SUMMARY

At temperatures above 80° F., mineral nutrition had little effect on the rust reaction of susceptible (Marquis), resistant (Mindum), or moderately susceptible (Thatcher) wheats. At temperatures between 65° and 75° F., Thatcher wheat was completely resistant to race 56 of *Puccinia graminis tritici* when supplied ammonium nitrogen and mesothetic, but approaching complete susceptibility, when supplied equivalent amounts of nitrate nitrogen. The response of Thatcher treated with ammonium nitrate was between these extremes. Mindum wheat was resistant regardless of nitrogen source.

Resistant and mesothetic leaves of Thatcher and resistant leaves of Mindum were analyzed for total, protein, amide, amino, and ammonia nitrogen, as well as for total soluble carbohydrates and reducing substances. The results are discussed in relation to existing theories of rust resistance in wheat.

Nitrogen source did not influence the rust reaction of Marquis, Mindum, or Thatcher wheat in the field.

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A METHOD FOR THE SELECTIVE STAINING OF VIRUSES IN INFECTED PLANT TISSUES[1]

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INTRODUCTION

The method of fixing and staining viruses in infected plant tissues described in this paper was evolved from trials with tobacco mosaic virus in tobacco. This virus produces three kinds of inclusion bodies in the parenchymatous cells of its host plant (11), two of which have been shown by inoculation to contain high concentrations of virus.

The criteria of successful staining demanded of this method applied to tobacco mosaic were (1) that all three types of inclusion body should stain in the same way, and (2) that samples of purified tobacco mosaic virus *in vitro* should react to fixation and staining in much the same way as the inclusion bodies in the tissues. This paper outlines the development of the method, and suggests a staining schedule that has been widely tested on tobacco mosaic and other viruses.

PRELIMINARY STAINING OF PURIFIED VIRUS AND INFECTED TISSUES

Spot tests with purified virus. Spots of purified tobacco mosaic virus were dried on slides at 40°–45° C. from drops of a concentrated suspension in water. They were subjected to various treatments designed to expose the ribonucleic acid fraction of the virus (6) to the action of dyes. After the spots were submitted to these treatments, Giemsa stain was applied to them in order to demonstrate the retention or location of the virus ribonucleic acid. Giemsa has been used (5, 8, 9) as a specific stain to trace the location of ribonucleic acid in animal cells.

Two treatments designed to liberate the ribonucleic acid to the action of stains were: (a) Virus spots were treated with Lugol's[2] iodine and washed thoroughly in a 0.5 per cent solution of sodium thiosulphate, followed by distilled water. The treated spots gave a strong Millon's reaction.[3] (b) Virus spots were treated quickly with concentrated acetic acid instead of iodine, until they gave a weak Millon's reaction or none. If the treatment with acid was prolonged beyond a minute or two, the Millon's reaction was invariably negative. The negative Millon's reaction indicated that the protein fraction of virus had been split off and altered or leached away. The ribonucleic acid fraction, which is insoluble in concentrated acetic aid (6), was assumed to be left on the slide. When Giemsa stain was applied,

[1] This work was undertaken after consultation with Dr. T. E. Rawlins, Division of Plant Pathology, University of California. Dr. Rawlins first suggested the approach to the problem of staining plant viruses outlined below.

[2] Made up by dissolving 6 gm. of potassium iodide and 4 gm. of iodine in 100 cc. of water.

[3] This reaction was stronger than that given by the un-iodized control.

whereas the untreated spots stained weakly, the iodine or acetic acid treated spots stained strongly (unless the dilution of Lugol's iodine had been considerably greater than 9:1). The color was blue, sometimes with a reddish or purple tinge. If the iodine was not washed out of the spot the stain was purple.

When iodine dissolved in absolute alcohol was applied and the spots were washed with 0.5 per cent sodium thiosulphate in 50 per cent alcohol solutions before staining with Giemsa, the spots were colored a somewhat lighter blue. If the iodine was not washed out, the color was pink to red. Generally the alcohol seemed to oppose the action of the iodine, presumably because it is a mild reducing agent and the iodine a mild oxidizing agent, or because of the solvent action of alcohol on iodine.

Thus, the staining reaction was similar after treatment in aqueous solutions of iodine-potassium iodide which only slightly altered the virus, and concentrated acetic acid, which presumably leached away the protein fraction (6). It is likely that the ribonucleic acid fraction of the virus took up much of the stain in the iodine-treated material.

Preliminary trials with fixed material. These were made mainly with pieces of tobacco leaves infected with tobacco mosaic virus, fixed in Karpechenko's fluid, embedded in paraffin, and sectioned at 10 or 12 μ. The sections were pretreated with Lugol's iodine or with a 2 per cent solution of iodine in absolute alcohol. They were then treated either with Giemsa's or Wright's stain. These two methylene-blue-eosin combinations gave similar results. Orange G in clove oil was generally used as a counter stain after differentiation in alcohol.

The main feature of these trials was that the striate material, derived by fixation from the crystalline inclusions of tobacco mosaic (3), stained a strong purple when pretreated with Lugol's iodine and washed with sodium thiosulphate. The color was like that obtained in spots of purified virus treated with iodine-potassium iodide, which had not afterwards been washed with sodium thiosulphate. Virus in the cell, or associated cell constituents, appeared to retain iodine more strongly than did similarly treated purified virus *in vitro*. Further, when the tissue sections were pretreated with iodine in absolute alcohol and washed with sodium thiosulphate in 50 per cent alcohol, the striate material stained pink, a color fainter but similar to that produced in spots of purified virus pretreated with iodine in alcohol and containing residual iodine.

In both instances amoeboid x-bodies stained much the same color as the chloroplasts, but more strongly. When the counter-stain was orange G, the chloroplasts, amoeboid x-bodies, and nucleoli were green, the chromatin of the nucleus was blue, and the cell walls faintly yellow. The purple color of the striate or crystalline material stood out very clearly.

In control sections of infected tissues not treated with iodine, the striate material had a trace of the yellow color from the orange G, but was only

faintly visible, and the x-bodies were rather lightly stained. In sections of healthy leaves pretreated with iodine the colors of the normal cell constituents were similar to those of the same structures in diseased leaves, except that sometimes the plastids stained somewhat less intensely than those in diseased tissues.

A TECHNIQUE OF FIXING AND STAINING

Methods. After the preliminary trials, the material adopted for tests of fixing and staining methods was epidermal strips, mainly from the leaves

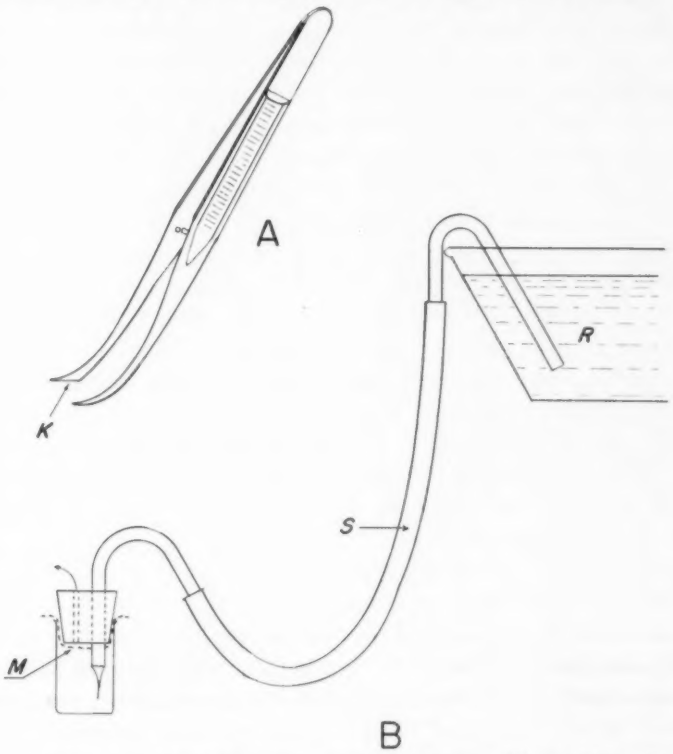

FIG. 1. A. Pair of stainless steel forceps prepared for making epidermal strips. The curved edge of one arm is ground on the outside to a knife edge (K). B. Siphon arrangement for washing epidermal strips and other material. Tap water is siphoned from reservoir (R) through tube (S) into a vial containing the material to be washed. The outlet from the vial is a hole in the stopper, covered by muslin (M).

of tobacco plants, healthy or infected with tobacco mosaic. Other hosts and other viruses were also tested, and some sections made by hand or freezing microtome were fixed and stained. Several lots of material were fixed and embedded in paraffin. The convenience of the epidermal strip technique and the ease and speed with which tests can be made were the reasons why it was most often used in fixing and staining trials.

Following are some of the details of the technique finally adopted. The epidermal strips were taken from the undersides of leaves, or from petioles

or stems. The instrument used was a pair of stainless steel forceps with smooth curved points. The lower outside edge of the arm further from the operator was ground to a knife edge and slightly turned so that it fitted smoothly against the curved end of the nearer arm (Fig. 1, A). The knife edge was pushed into the junction of two veins on the underside of a leaf or under the epidermis, the forceps closed, and a piece of the epidermis gently stripped off. Often very turgid leaves were easily stripped; if not, before stripping, they were allowed to wilt slightly, but not too much in case severe wilting affected the cell contents. When even wilted leaves could not be stripped, epidermis was sometimes stripped from petioles or stems. To prevent wound reactions in the epidermal cells, stripping was done under the fixative in a flat dish. Surgical rubber gloves were sometimes used to protect the fingers. Some trials were made stripping the epidermis under isotonic sugar solutions, but they were insufficient in number to determine whether this would prevent the secondary effects of wounding on the structure of intact cells. The epidermal strips were placed immediately in fresh fixative contained in small glass vials.

Washing with tap water when it was needed was done by siphoning from a flat dish into which water flowed directly from a tap (Fig. 1, B). The size of the siphon inlet and the level of the outflow from the vial determined the rate of flow, and were easily regulated. The delivery into the vial occurred a little more than half way down its length through a glass tube carried in a rubber stopper. The outlet hole through the stopper was covered by muslin (Fig. 1, B). To wash epidermal strips in a vial, water was started through one of the siphons, the end was plugged into the top of the vial, and the vial was placed in a test tube stand at a height that insured a desirable rate of flow.

A device used for staining paraffin sections on slides was the ring of a van Tiegham cell accurately ground to fit on the flat surface of the slide. The ground surface was dipped into glycerine, placed on the slide around the sections, and gently pressed to the surface. Stains dissolved in volatile liquids such as methyl alcohol can be held in this container for some time if it is covered with a cover slip. The ring can be lifted several times to allow a solution to run off, the sections can be washed, and the ring replaced and refilled with another solution.

Fixation and staining. The first fixative tried was simply Lugol's iodine diluted with an equal volume of absolute alcohol containing 2 per cent iodine. The fixative of this type adopted after a number of trials contained less iodine and some formalin, the formalin being added just before fixation was begun. The proportions were:

Lugol's iodine (6 gm. potassium iodide, 4 gm. iodine, 100 cc. water)	20 cc.
Water	25 cc.
Alcohol	50 cc.
Formalin (38–40 per cent formaldehyde)	5 cc.

This was designated fixative 1a.

The reasons for adopting this formula were: 50 per cent alcohol alone precipitates the virus; iodine alone, when it oxidizes the SH radicle of the virus, renders it insoluble (1); and the initial pH value of the mixture, about 3.6, is near the isoelectric point of purified virus and within the range where complete precipitation of the virus occurs in presence of cell components (2). This fixative appeared to maintain the virus inclusions of tobacco mosaic in much the same condition as in the living cells. Crystalline inclusions in recently infected tissues often were whole and without any sign of striations. Others were cracked, sometimes in mosaic patterns, but this may have been an effect of dehydration rather than of fixation. Virus which had apparently been diffused throughout the cytoplasm of the living cell often appeared to be fixed in disperse form, and could be stained the same color as the more concentrated inclusion bodies.

Formalin improved the fixation of cell contents, and a 1 in 20 concentration (2 per cent formaldehyde) was not high enough to interfere seriously with the oxidizing action of the iodine on the virus. Increasing the concentration to 1 in 10 without increasing the concentration of the iodine did interfere with the action of the iodine, and resulted in poorer staining of the virus. When the mixture was made up with 1 in 20 formalin neutralized by calcium carbonate, the pH value within a few minutes of preparation was around pH 5.2, and during a period of about 30 min. it fell to about 4.2. Presumably because the oxidation-reduction potential also was affected, inclusions in infected tissues fixed in this mixture failed to take up Giemsa stain, appearing like those in tissues fixed by standard methods.

The main disadvantage of this fixative for paraffin material is that, lacking the swelling component, acetic acid, it may be followed by shrinkage of the cells if it is not carefully handled, or the tissues may become brittle. Where even shrinkage of tissue components can occur, it is capable of producing useful results, for example, in leaves and leaf primordia; but where even shrinkage is not possible it may cause distortion of cell walls and cell contents. Also, the cytoplasm is flocculated, and the plastids may appear empty except for the starch grains.

This fixative may be followed by a Giemsa and orange G combination of stains, or iron alum haemotoxylin, trypan blue, etc. Only the Giemsa schedule is contained in this paper. Giemsa and orange G have given selective staining of virus in epidermal strips, but have been found somewhat more difficult to control when used on paraffin sections of growing tips.

The two stains may be applied together as a mixture. If they are, the orange G is prepared and kept separately as a 3 per cent solution in the same solvent as Giemsa, 3 parts methyl alcohol and 1 part glycerine. The stains are mixed immediately before application. They may be used undiluted or diluted 1 in 3 with methyl alcohol. The proportions have varied from 1 volume of orange G and 2 volumes of Giemsa to 1 volume of orange G and 19 of Giemsa. When the proportion of orange G is low, the staining

time may be lengthened and an intenser color obtained without too much displacement of the Giemsa dyes by the orange G.

The mixture of dyes, undiluted or diluted with methyl alcohol, is applied to the epidermal strips or sections in a vial for a period of from one to ten minutes. It is then diluted drop by drop with an equal volume of M/50 phosphate buffer of pH 7 (4, Table 41), and thereafter is left on the preparation for the same time or somewhat longer. The proportions and timing suitable for the material under examination may be found by a few preliminary trials.

In the resulting preparations of epidermal strips, the amoeboid bodies, as well as other virus inclusions, stain purple and the host nucleus stains greenish-blue to blue. Unlignified cell walls, for the most part, are barely outlined. The terminal cells of leaf hairs, even of healthy plants, may contain some purple material if the stains are not correctly balanced; but if they are, the contents of these cells, in the absence of virus, are generally green. The thick walls of guard cells may be blue-green or blue, and in sections, the walls of vessels are a light green. In good preparations of epidermal strips no purple-staining cell constituents have been found apart from those that can be interpreted as virus, except that sometimes the nucleoli are also purple. Nucleoli are said to contain ribonucleic acid (See (5) for a summary of information on this point). If the Giemsa is used alone, more intense staining is obtained, but other cell structures besides virus may also stain purple, or blue may be the predominant color throughout.

A staining schedule that was applied successfully by students during a demonstration of these fixation and staining methods may be given as a working example. This schedule was not designed to preserve the leaf hairs, most of which collapse during the transfer from acetone to xylol, or xylol to balsam. The hairs may be preserved by increasing the period of fixation, hardening in 95 per cent alcohol, and using a graded series of acetone-xylol mixtures and an infiltration process for the change to balsam.

1.	Fix	30 min. or longer
2.	Wash in sodium thiosulphate solution, 0.25 per cent in 50 per cent alcohol	10 min.
3.	50 per cent alcohol, two changes	10 min.
4.	70 per cent alcohol	5 min.
5.	95 per cent alcohol	20 min.
6.	Giemsa stain, 4 parts, orange G, 1 part (0.2 to 0.5 ml.)	2 min.
7.	Dilute drop by drop with an equal quantity of M/50 phosphate buffer, pH 7	3 min.
8.	Rinse, Lugol's iodine 1 part in 99 parts of 80 per cent acetone	20 seconds or less
9.	Absolute acetone, three changes during	5 min.
10.	Xylol	5 min.
11.	Mount in neutral Canada balsam.	

The chief danger during differentiation (step 8) is of leaching out too much stain. Less rapid differentiation may be accomplished by passing the tissues rapidly through 70 per cent and 85 per cent acetone, or even directly to acetone. The more gradual the transition from stain to acetone the less the shrinkage of the epidermal strips; the more rapid, the stronger the staining. If the epidermal strips are washed thoroughly after fixation, and the stain is differentiated without iodine, virus may appear blue rather than purple. This may at times be considered desirable. A workable compromise for any particular purpose or lot of material can be discovered by trial.

SUMMARY

1. Spot tests with purified tobacco mosaic virus proved that pretreatment with iodine-potassium iodide solutions or concentrated acetic acid facilitates the staining of the virus with Giemsa stain.

2. Sections of tobacco leaves diseased with tobacco mosaic and fixed in Karpechenko's fluid were pretreated with iodine-potassium iodide solution and stained with Giemsa-orange G. The virus in the form of striate material stained purple.

3. A fixing and staining schedule was evolved on this basis that gave selective staining of tobacco mosaic virus inclusions and other more disperse material, presumably virus, in the cytoplasm. The test material was mainly epidermal strips from infected tobacco plants, and from normal controls.

ACKNOWLEDGMENT

The results contained in this and in three succeeding papers were obtained while I was working as a visitor in the Division of Plant Pathology, University of California, Berkeley, California. I wish to thank both the Council for Scientific and Industrial Research, Australia, for making this period of work possible, and members of the University of California Plant Pathology staff, particularly Dr. M. W. Gardner and Dr. T. E. Rawlins, for laboratory accommodation, material, and constant help in many forms.

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DEVELOPMENT OF THE LEAF SPOT FUNGUS IN THE OLIVE LEAF[1]

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(Accepted for publication January 17, 1949)

Boyer (2) in 1891 gave the first detailed description of the olive leaf spot fungus, *Cycloconium oleaginum* Cast. He noted its presence on both surfaces of the leaf, on the peduncle of the fruit, and very rarely on the fruit itself. He sectioned diseased leaves and described the growth of the fungus in the leaf cuticle. Brizi (3) in 1899 described the morphology of the fungus, illustrated the changes which it produced in the leaves of the olive, and investigated its life history extensively. Ducomet (5) in 1907 and Petri (7) in 1913 studied the development of the mycelium of *C. oleaginum* within the olive leaf and compared it with other intracuticular organisms.

Histological studies were made by Cavadas (4) in 1925, who reported that the mycelium penetrated all the tissues of the leaf by very fine prolongations.

Histological studies of the lesions in living leaves were made by the writer in an attempt to correlate growth of the fungus in the tissue with symptom development and conidial production. Other studies were concerned with the development of the fungus in fallen leaves.

MORPHOLOGY OF THE EPIDERMIS OF THE OLIVE LEAF

Petri (7) described the cuticle of the olive leaf as composed of five distinct layers: an outer, very thin wax stratum, the cuticle proper, two inside cuticular strata, and the layer of epidermal cells. He maintained that the fungus *Cycloconium oleaginum*, growing between the two cuticular layers, secretes enzymes capable of dissolving the cutin, which it utilizes as a source of energy.

To determine the morphological structure of the epidermis, microchemical and optical tests of sections of healthy olive leaves were made by the methods described by Rawlins (8). The leaf material was killed in formalin-acetic-acid-alcohol mixture, embedded in paraffin, sectioned 12 to 14 μ thick, mounted on slides, and treated with xylene and alcohol.

A cross section of a healthy mature leaf, treated with zinc-chlor-iodide or iodine-potassium iodide followed by 65 per cent sulfuric acid, revealed four differently stained layers within the cuticular and epidermal region. The outer, very thin dark wax layer and beneath it the cuticle proper, which

[1] Part of a thesis submitted to the Graduate Division of the University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Associate Plant Pathologist in the Experiment Station, University of Florida, Gainesville.

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together averaged 3 μ thick, stained yellow. Under the cuticle was a layer which stained dark orange to brown, and below this the innermost layer, composed of epidermal cells which stained blue in the regions of the cell walls.

Cross sections of healthy leaves were mounted on slides in water and studied with polarized light. When the section was rotated at an angle of 45° with the vibration axis of the analyzer, three anisotropic tangential layers were visible within the epidermal region (Fig. 1, A). Rotation of the field to the 90° angle position showed anisotropic radial layers extending

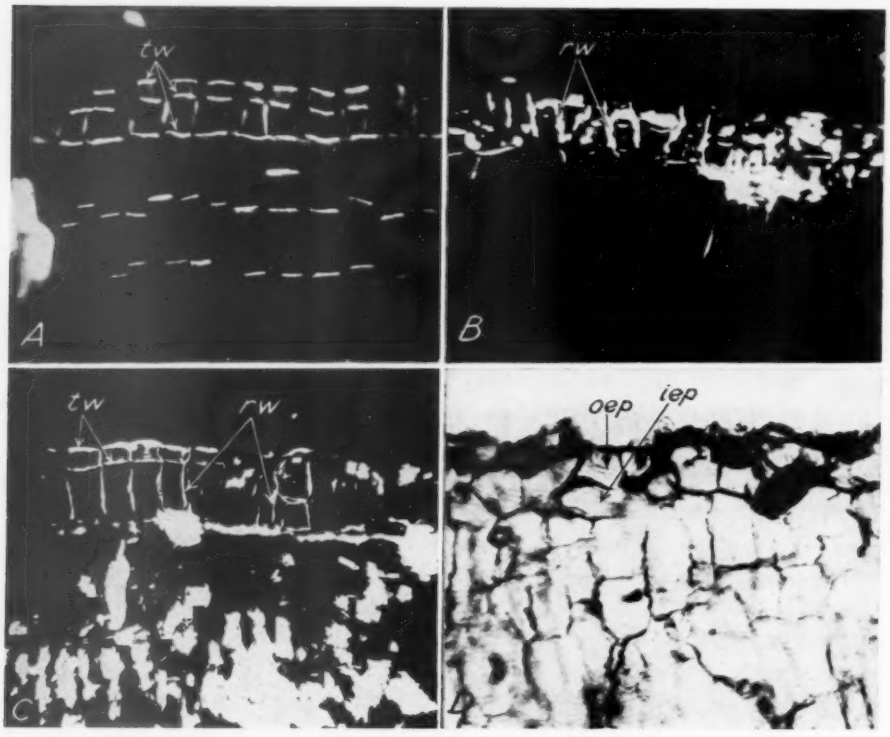

FIG. 1. Cross sections through the epidermis of a normal mature olive leaf (all ×270). A. Photographed under crossed Nicol prisms with the section rotated at an angle of 45° with the vibration axis of the analyzer to show the tangential walls (tw) of the two epidermal cell layers. B. Photographed with field rotated to the 90° position to show radial walls (rw) of the epidermal cells. C. Taken at a plane of rotation to show some tangential and radial walls of the two layers of epidermal cells. D. Section treated with KOH to remove cutin and waxes, then stained with Delafield's hematoxylin to show the two layers, (oep) outer epidermal cells and (iep) inner epidermal cells.

from the lower edge of the epidermis to the layer of cuticle (Fig. 1, B). These doubly refractive layers were regarded as the tangential and radial walls of two layers of epidermal cells. The anisotropy of the layers probably results from the presence of cellulose, because cellulose is doubly refractive or anisotropic when viewed between crossed Nicol prisms.

The photograph in figure 1, C, was taken at a plane of rotation between crossed Nicols in which some radial and some tangential walls of the epi-

dermal layers were anisotropic. These observations indicate that the layer which lies just beneath the cuticle proper, and which stains dark orange or brown with zinc-chlor-iodide, is cellular in nature and constitutes a second layer of epidermal cells above the layer easily recognized as epidermis. Failure of the walls in this cell layer to stain blue with a cellulose stain is due to their impregnation with cutin. The cutin probably accounts for the fact that the cellular nature of this epidermal layer is not clearly apparent upon examination with the ordinary microscope.

Attempts were made to remove the cutin and other fatty materials from the epidermis of the leaf sections. The cutin was insoluble in ether and benzene. After the sections were submersed in ether for 24 hr. some slight clearing occurred within the outer layer of epidermal cells. In sections autoclaved in alcoholic KOH at 15 lb. pressure for one hour, varying degrees of clearing occurred, and in thin sections almost complete hydrolysis of the cutin and fatty materials had taken place. The cleared sections were washed in water for 30 min. and then stained with Delafield's hematoxylin. The walls of the two layers of epidermal cells remained intact although somewhat distorted by the treatment (Fig. 1, D).

These studies indicate that the epidermis of a mature olive leaf is composed of an outer thin wax layer, a layer of cutin (hereafter called the cuticle proper), and two layers of epidermal cells, the outer impregnated with cutin and fatty materials. The cells of the two layers are not similar, those of the innermost layer being elongated radially. The cell walls in the two layers may or may not occupy parallel planes.

INITIATION OF INFECTION AND GROWTH OF THE FUNGUS IN LIVING LEAVES

Information on the time of year when natural infection occurs and symptoms develop was inadequate. Petri (7) maintained that the fungus develops mainly at the end of summer and the beginning of spring. Hayne (6), who first reported on the disease in California, stated that the fungus appears on the leaves in any season of the year, and that lesions in all stages of development sometimes are found on the same leaf. He stated further, however, that the disease usually makes its appearance at the end of summer or in autumn. In the winter of 1946–47, Wilson and Miller (9) studied infection periods in central California by exposing small potted olive trees in an orchard under leaf-spot-infected trees for a time and then removing them to a lath house. With favorable moisture and temperature, infection was initiated at various times between late November and late February, but in none of these particular tests did lesions become visible until early spring. Judging from these data, conidia of *Cycloconium oleaginum* germinate at temperatures between 9° and 25° C., with the optimum near 16° C.; and the mycelium grows at temperatures between 12° and 30° C.

To study the growth of the fungus in living leaves, infected material was collected at regular intervals over a twelve-month period. Pieces cut from the lesions were killed in formalin-acetic-acid-alcohol mixture and embedded in paraffin.

For very young lesions, before the hyphae of the fungus had assumed the dark brown color typical of older mycelium, cotton blue was the most satisfactory stain. After staining, the sections were differentiated with 95 per cent alcohol and cleared in clove oil. Where greater differentiation between the fungus and the cellular structure of the leaf was desired, a combination of safranin and fast green or of bismark brown and methyl violet was used. Staining with Sudan IV revealed the path of the fungus hyphae through the cuticle. The mycelium and spores in old lesions, were dark brown to brick red in advanced stages of development and could be seen and studied easily without the use of stains.

It has been assumed that germ tubes of *Cycloconium oleaginum* conidia pass through the leaf cuticle, but the writer was unable to find a germinated conidium on the leaf surface with germ tube penetrating the cuticle. Petri (6) maintained that the hyphae of *C. oleaginum* secrete enzymes which dissolve the cuticle in the immediate region of penetration.

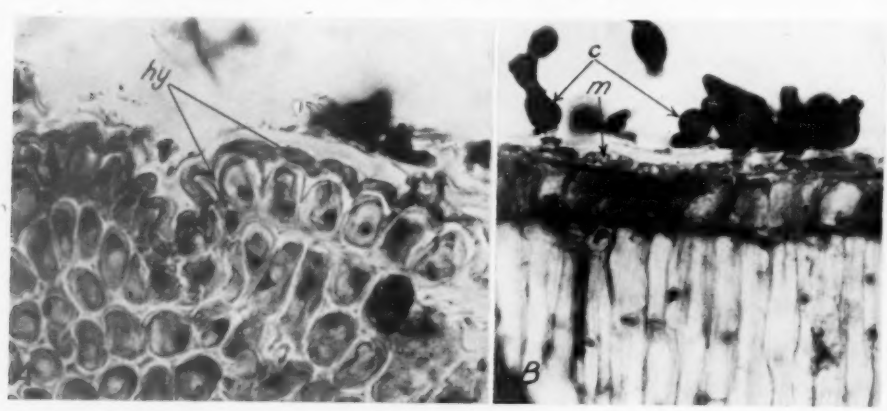

FIG. 2. Cross sections of Cycloconium-infected olive leaf (both × 270). A. Hyphae (hy) within the cells of the outer epidermal layer. B. Mycelium (m) within the cells of the outer epidermal layer and conidia (c) on the leaf surface. The cellular nature of the epidermal layer which the fungus occupies is not evident from the picture, because the outer layer of the epidermal cells is impregnated with cutin or fatty materials which prohibit absorption of the stain used.

In an area where infection had occurred recently, hyphae were abundant in the outer layer of epidermal cells. These hyphae had developed tangentially within the epidermal cells of the upper layer and were both intracellular and intercellular (Fig. 2). They were light brown, uniform in diameter, and continuous for considerable distances. The mycelium layer in the outer epidermal cells was one hypha thick, and followed closely the surface contour of the epidermal cells except where numerous hyphal branches arose and penetrated the cuticle to the outside. These external hyphae bore conidia directly or expanded into bulbous conidiophores on which the conidia were borne. At this stage of development the mycelium in the leaf had not penetrated below the first layer of epidermal cells. No breakdown in or injury to the cells of the leaf was apparent. It is assumed

that the nutrients required for fungus growth are obtainable in this area of the epidermis.

Lesions, when first visible, are very small, brown sooty blotches on the leaf surface. Leaf pieces with lesions in this stage of development were cleared by boiling in 70 per cent alcohol to remove the chlorophyll and then were boiled in 5 per cent sodium hydroxide. Microscopical examination of the cleared leaf pieces showed the presence of some mycelium on the surface. The scattered hyphae were composed of short, thick, dark brown cells. The sooty appearance of the lesions, however, was due in large measure to the numerous conidia.

Leaf lesions which appeared in early spring continued to enlarge and develop during April and May, and often coalesced to cover a considerable area of the leaf surface. Color of the lesions did not change during this period, because the sooty brown to black spores were abundant.

During the summer the mycelium became darker brown and more septate. The cuticle and outer walls of the epidermal cells of the leaf became thicker, as did the mycelial layer within the outer layer of epidermal cells; and prolongations of the hyphae extended between the radial walls of the inner layer of epidermal cells. No light-colored hyphae, characteristic of actively growing fungus, were found. Such a condition is consistent with the disease picture, inasmuch as growth and expansion of the leaf lesions apparently cease during the hot, dry summer months.

Severely infected leaves fall during late spring and early summer. On leaves remaining on the tree during the summer, the yellowed leaf area surrounding a lesion was a striking contrast to the brown lesion with its series of more or less concentric zones. Slowly developing lesions remained as gray-to-black spots on a green leaf and failed to produce conidia.

In summer, moreover, the lesions became dry, hardened, cracked or blistered, and scarlike. The cuticle broke away from the epidermal cells and in the center of the spot the outer epidermal cell walls sloughed off. This effect was apparently produced by the fungus since the mycelium had grown throughout the outer epidermal cells. Other hyphae had grown between cells of the inner epidermal layer and along the end walls of the palisade cells, in many cases isolating single cells. Only rarely in this stage of development did the fungus grow into the lumen of the cells of the inner epidermal layer or into the palisade cells. Some of the epidermal cells were filled with a light brown granular material which failed to take the stains used.

In autumn, some of the lesions on leaves that had remained on the trees expanded at their margins. These margins of newly involved tissue were brown and bore abundant conidia. New mycelium that had extended from the periphery of old lesions into adjacent tissue had much the appearance of mycelium found in early stages of lesion development. The new hyphae extended singly and tangentially along the outer layer of epidermal cells, and numerous sporophores arose from these hyphae, passed through the

cuticle to the outside, and bore conidia at the surface of the leaf. These expanded lesions were an important source of inoculum for new infections in late fall (9). However, such an expansion of old leaf spots did not always occur.

Many small lesions over which the cuticle of the leaf had not disappeared during the summer again became active during autumn and produced spores over the entire surface of the lesions; these likewise provided a supply of spores for new infections. Such lesions apparently were initiated in late spring, and before they had developed to any great extent their growth was arrested, probably by high temperatures.

So far as was known there was no other effective source of inoculum (9). The old lesions which resumed growth in the fall of the year probably provided an adequate supply of conidia to account for subsequent infection. However, some workers believed that fallen leaves on the ground might be

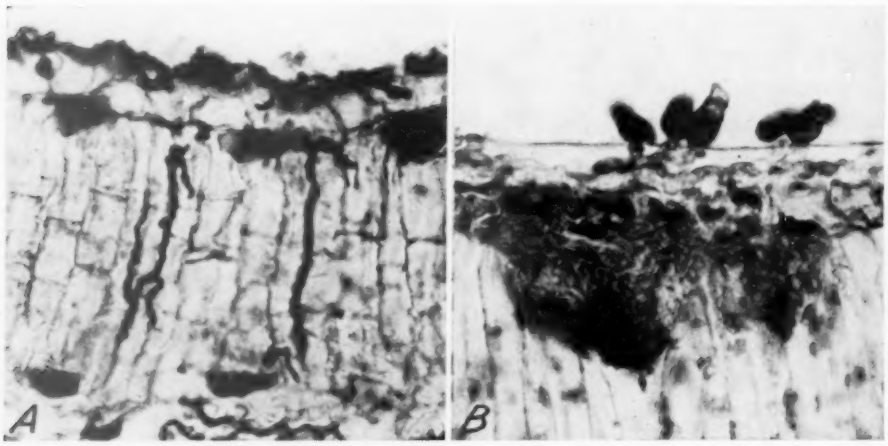

FIG. 3. Cross sections of dead olive leaf invaded by Cycloconium (both × 270). A. Mycelium growing throughout the epidermal cells and hyphae extending down between the palisade cells. B. Stromatic mass of fungus cells growing in the tissue of the leaf. Typical conidia of *Cycloconium oleaginum* on hyphae growing out of the fungus tissue.

a source of inoculum. Bernes (1), for example, recommended burning the leaves as a control measure.

GROWTH OF THE FUNGUS IN DEAD LEAVES

To study the behavior of the fungus in fallen dead leaves, affected leaves were collected from the ground in the spring of the year and at various times throughout the late fall and early winter. Some of the leaves had been on the ground for several months while others had fallen recently. Judging from microscopical examination of sections of such material, the fungus did not die when the leaves fell, but remained alive for some time growing as a saprophyte. The behavior of the fungus in dead leaves differed markedly from that in living leaves in that the mycelium ramified throughout the leaf tissue even to the lower epidermis (Fig. 3, A). In the leaves that had not

been on the ground long enough to disintegrate and to be overrun by other fungi, conidia of *Cycloconium oleaginum* were produced in abundance. The time of this conidial production corresponded with that on leaves in the trees.

Although examination of numerous sections failed to show a sexual stage of the fungus, certain stromatic bodies were found (Fig. 3, B). The significance of these in the life history of the organism was not ascertained. They were composed of a basal portion of pseudo-parenchymatous mycelium which was embedded deeply in the palisade tissue of the leaf, and a top portion in the subcuticular region of the leaf composed of closely entwined hyphae. In many cases, sporophores arose from this mycelial layer, passed through the cuticle, and produced conidia typical of *Cycloconium oleaginum*. In other instances the stromatic mass had greatly expanded and broken through the leaf tissues to the outer surface. Since other fungi grew abundantly on the dead olive leaves, it is difficult to state definitely that the stromatic bodies were structures of *C. oleaginum*. Such bodies were found in leaf lesions which appeared to be free of any fungi, and hyphae and conidia typical of *C. oleaginum* were closely associated with them. They developed during the saprophytic life of the fungus or when the fungus was subjected to adverse conditions. No similar structures have been found associated with the fungus on living leaves. Furthermore, Wilson and Miller (9) found similar stromatic tissue in pure cultures of *C. oleaginum*.

DISCUSSION

Cycloconium oleaginum has hitherto been considered an intracuticular organism which by the secretion of enzymes is able to utilize the cutin as a source of energy. The results of the present investigation demonstrated that the epidermis of an olive leaf is composed of two layers of epidermal cells and that the fungus grows in the cells of the outer layer. Consequently, the fungus probably utilizes materials within such cells as food sources.

Initial infection and development of symptoms of the olive leaf spot can be correlated with growth of the fungus in the leaf tissue. In late fall, winter, and early spring when conditions favor infection, the fungus is expanding in the leaf and producing abundant conidia. The fungus is more or less inactive during the summer. It survives in many of the leaves which remain in the tree, resumes its growth in late fall, and again produces conidia.

In dead leaves on the ground, *Cycloconium oleaginum* may remain alive for some time, undergoing extensive saprophytic development. Conidia may be produced in abundance on these leaves. It is doubtful, however, if these spores are an important source of inoculum. Judging from field observations (9), conidia of *C. oleaginum* are carried short distances by the wind or are disseminated in water droplets which are spattered around. In a dry state the conidia are not easily detached from the conidiophores by air currents and, for this reason, perhaps are not widely disseminated by wind.

SUMMARY

The epidermis of a mature olive leaf is composed of an outer thin wax layer, a layer of cutin, and two layers of epidermal cells. The upper layer of epidermal cells is embedded in cutin and other fatty materials which can be removed by autoclaving in alcoholic KOH. The walls of this layer of cells possess the same refractive index as the cutin, fail to take the cellulose stains, and are not visible in sections until the cutin is removed. The cell walls are visible, however, when viewed in polarized light.

The mycelium of *Cycloconium oleaginum* develops one-hypha-thick in the outermost layer of epidermal cells and is both intercellular and intracellular. Short hyphal branches arise along the mycelium, grow through the cuticle to the leaf surface, and produce single spores of one or two cells.

Growth of the fungus in the leaves during the early summer months proceeds at a slow rate. The mycelium ramifies through the leaf tissue and between the cells of the inner epidermal layer. No conidia are produced during the summer.

In the fall of the year, some of these inactive lesions expand and produce spores on their margins. Other lesions of more recent origin do not expand, but become active and produce spores over their entire surface.

The fungus remains alive and continues to grow as a saprophyte on olive leaves after they have fallen to the ground. Here it develops extensively in the host tissue and, for a time after the leaf falls, produces conidia.

No perfect stage was found in these studies, but certain stromatic bodies associated with the fungus were found in dead olive leaves bearing old lesions.

Apparently infection and development of lesions are influenced by moisture and temperature and, in California, may occur at any time during fall, winter, or early spring when such conditions are favorable.

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PHYTOPATHOLOGICAL NOTES

Parasitism of Citrus in Florida by Various Species of Dodder, Including Cuscuta Boldinghii Urb., a Species Newly Reported for the United States. Cuscutas on citrus have been reported in the past, but in the United States only George[1], Stout[2], and Bellue[3] have listed specific names of the dodders involved. George's report mentions the attack of citrus in Arizona by *Cuscuta gronovii latiflora*, and Stout's and Bellue's reports mention the parasitism in California of *Citrus sinensis* (L.) Osbeck by *Cuscuta subinclusa*. An emendation is suggested by Dr. Freeman Weiss[4] to the reference in his Check List Revision to *Cuscuta americana* L. as a parasite of *Citrus* spp. in California and Florida. According to Dr. Weiss, the record of this dodder, originally made by Nowell in Diseases of Crop Plants in the Lesser Antilles, should have been restricted to the West Indies where this species is endemic. Lacking reports of an identified species on citrus, the record for the states above mentioned, including Arizona, should have read *Cuscuta* sp. indet.

Abroad, specific determinations of dodder affecting citrus are limited to the reports of Gandara[5] in Mexico and Nowell[6] in the Lesser Antilles, who mention orange and lime respectively as being parasitized by *Cuscuta americana*.

In Florida the writer has observed three species of dodder on citrus and a near-citrus species: (1) *Cuscuta americana* L. on *Citrus sinensis* (L.) Osbeck (sweet orange), *Citrus paradisi* Macf. (grapefruit), *Citrus Limon* (L.) Burm. (lemon), and *Citrus aurantifolia* (Christm.) Swingle (Tahiti lime); (2) *Cuscuta campestris* Yunck. on *Fortunella margarita* (Lour.) Swingle (Nagami kumquat); and (3) *Cuscuta Boldinghii* Urb. on *Citrus sinensis*.

The above three dodders were identified through the courtesy of Dr. T. G. Yuncker, De Pauw University. Concerning *Cuscuta Boldinghii*, he writes as of September 29, 1948: "Your other specimen collected at Tavares is most interesting as it is the first report of this species in the U. S. A. It is *C. Boldinghii* Urb. The type was from Bonaire. I also have it from Curacao, Yucatan, Mexico, Haiti and Honduras . . . [as well as] from Guatemala and Costa Rica. . . . None of my former records report it on Citrus."

Regarding the incidence of these three species of dodder on Florida citrus, *Cuscuta Boldinghii* has been found at only one location, in Lake

[1] George, D. C. A parasitic dodder on citrus and olive. Arizona Comm. Agr. and Hort. Ann. Rept. **9**: 65–66. 1917.

[2] Stout, G. L. A case of dodder (*Cuscuta subinclusa* Durand and Hilgard) on Valencia orange (*Citrus sinensis* Osbeck) in Southern California. California Dept. Agr. Bul. **29**: 121–124. 1940.

[3] Bellue, M. K. Record of dodder (*Cuscuta subinclusa* Durand and Hilgard) on citrus in Orange County. California Dept. Agr. Bul. **29**: 145. 1940.

[4] Weiss, Freeman. Check List Revision, U. S. Dept. Agr., Pl. Dis. Reptr. **25**: 36–46. 1941. A letter from Dr. Weiss dated Oct. 11, 1948, gives the above correction.

[5] Gandara, G. Enfermedades y plagas del naranjo. Estac. Agr. Centr. Mexico Bol. **111**: 5–40. 1920.

[6] Nowell, W. Diseases of crop-plants in the Lesser Antilles. 383 pp. The West India Comm. London. 1923.

County, where it was growing mainly on the cover crop and only weakly on an overhanging branch of orange.

Cuscuta campestris, similarly, has been observed but once, in Polk County, growing but sparingly on Nagami kumquat.

Cuscuta americana, in contrast to the two foregoing species, is generally distributed, grows vigorously on citrus, and once established covers a tree with a matting so heavy and tenacious (Fig. 1) that its eradication becomes a matter of considerable cost. According to records of one fruit-producing company, the hand removal of dodder from a single tree last year entailed an expenditure of 48 man hours.—L. CARL KNORR, Citrus Experiment Station, University of Florida, Lake Alfred, Florida.

FIG. 1. Parasitism of *Citrus sinensis* by *Cuscuta americana.*

A Simple Power-Operated Atomizer for Applying Small Quantities of Concentrated Spray Fluids.—In the course of experimental work on tar acid compounds for end of season killing of potato vines[1], it became necessary to apply small quantities (10–50 ml.) of many different concentrated liquids to plots of six plants each. Hand-operated atomizers proved both slow and tiring to the operator and the necessity for repeated washing out of the container was another disadvantage. As the measured quantities of the sprays were carried to the field in $6 \times \frac{3}{8}$-in. boiling tubes it was decided to make a small atomizer of the scent spray type, using these tubes

[1] Wilson, A. R., A. E. W. Boyd, J. G. Mitchell, and W. S. Greaves. Potato haulm destruction with special reference to the use of tar acid compounds. Ann. Appl. Biol. 34: 1–33. 1947.

as interchangeable containers. As an automobile was usually available on the experimental field, a convenient source of pressure was obtained by leading the exhaust gases to the plots by means of a length of hose pipe. This system has proved so satisfactory that it seems likely to be of use to anyone engaged on similar work where contamination with exhaust gases and small quantities of condensation water is immaterial.

Two lengths of 1-in.-wide, light gauge, tinned iron are bent as shown in figure 1 to form a spring cradle for the container. The two halves are riveted together at (a) so that the bottom half (c) hinges on the top half (b). When the cradle is closed, the base of the container (d) is held in the

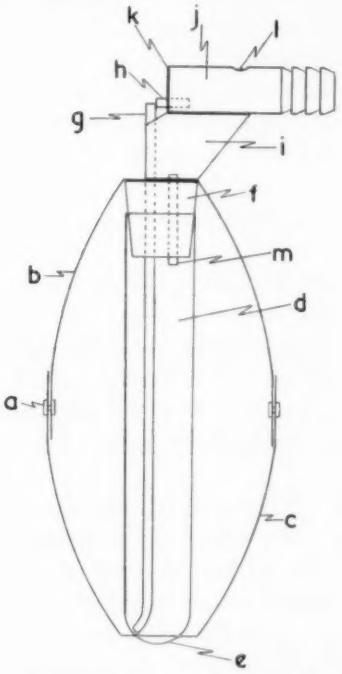

FIG. 1. Side elevation of atomizer (to scale).

depression (e) while the top is pressed firmly on to the rubber bung (f). The feed (g) and pressure (h) pipes are of $\frac{1}{16}$-in.-bore copper tubing firmly held at the correct angle to each other by two pieces of tinned iron (i) soldered, either side of the feed pipe, to the top of the cradle and to the underside of the hose connection link (j), respectively. The end of the hose connection link is blocked by a disk of tinned iron (k) through which is soldered the pressure pipe. A $\frac{1}{4}$-in. hole is drilled at (l) to serve as a pressure release valve. A short length of $\frac{1}{16}$-in.-bore copper tubing (m) serves the dual purpose of preventing the development of negative pressure in the container and stopping rotation of the bung on the feed pipe.

To operate the atomizer, the hose line from the exhaust is pressed on to the hose connection link and the thumb is held over the hole (l). Less than 0.5 ml. of spray fluid remains in each container after use.

Since the work described was completed, details of another apparatus designed for a similar purpose, but not on the atomizer principle, have been published.[2]—A. R. WILSON, Agricultural Research Council, Potato Storage Investigation, care of Nottingham University, School of Agriculture, Sutton Bonington, Loughborough, England, and A. E. W. BOYD, now at The Department of Agriculture for Scotland, Plant Pathology Laboratory, East Craigs, Corstorphine, Edinburgh.

A Method for Preserving and Mounting Pressed Leaves.[1] There has been a long-felt need for a simple and convenient method of preserving and mounting leaves without loss of color and detail. Also it is frequently desirable to be able to see both surfaces of a leaf or to view it with transmitted light, particularly if one wishes to study symptoms of virus or other diseases. Herbarium specimens, either in conventional mounts or unmounted, are not entirely satisfactory for class purposes, since dried leaves are too brittle for frequent handling and their distinctive markings are likely to fade or to disappear.

After considerable experimentation, the following method of mounting has been devised to overcome some of the aforementioned disadvantages. Leaves showing the desired characteristics are pressed and dried in the usual way as soon as possible after collection. At this stage, the use of heavy, corrugated cardboard interleaves has proven advantageous. The corrugations should run in the same direction in all interleaves, to facilitate air circulation through the press. The pressure exerted on the press should be evenly distributed and sufficient to permit the specimens to dry without wrinkling.

When the leaves are thoroughly dried, a coat of medium thin white shellac is applied with a camel's-hair brush to both surfaces. The shellacked leaves can be placed to dry overnight on a sheet of waxed paper or on a coarse wire mesh. The shellac has the effect of restoring the lustre and color of the leaf and of giving markings and patterns a remarkably natural appearance. The specimens are then arranged on the adhesive side of a sheet of glossy Permafilm[2] or similar preparation, and covered with a duplicate sheet applied according to the manufacturer's directions. The application of the covering sheet requires some patience and care in order to avoid wrinkling and air spaces where the films come in contact with each other. A mistake at this stage is irremediable. If air is entrapped between the leaf and the transparent sheet it can be removed by making a needle puncture at the edge of the leaf or at the end of the petiole and exerting pressure on the mount either by rubbing with a soft cloth or by means of a photographic

[2] Davis, J. F. A sprayer attachment for rapidly applying small quantities of spray materials. Jour. Amer. Soc. Agron. 39: 835–836. 1947.

[1] Contribution No. 966 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

[2] Permafilm is the trade name used by Denoyer-Geppert Co., Chicago, Ill. It is probable that other films of a similar type are also obtainable.

print roller. The resulting transparent mount can then be trimmed to size. Leaves in this mount are reasonably flexible and can be handled safely without danger of breakage. The transparent portions may be silghtly cloudy at first but the cloudiness disappears in the course of a few days.

Mounts measuring, when trimmed, 4–7/8 × 7–3/4 in. can be made satisfactorily and are more easily filed than larger sizes. They can be attached to 5 × 8-in. filing cards. This can be done by laying on a table the card and the mount face up and end to end, separated by a 1/8-in. gap. Juncture is made with 1-in.-wide strips of Permafilm applied both back and front. The mount can then be folded back on the card, ready for filing. A description of the specimen or other notes can be typed on the face of the card before attaching the mount.—R. S. WILLISON, Box 28, St. Catharines, Ontario, Canada.